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Biochemical and Pharmacological Studies on the Interaction of Protamine with Heparins

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BIOCHEMICAL AND PHARMACOLOGICAL STUDIES ON THE INTERACTION OF PROTAMINE WITH HEPARINS

by

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VITA

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PUBLICATIONS

Racanelli, A., Fareed, J., Walenga, J.M. and Coyne, E.: Biochemical and Pharmacologic Studies of the Protamine Interactions with Heparin, Its Fractions and Fragments. Seminars in Thrombosis and Hemostasis, 11:(2) 176-189, 1985.

Racanelli, A., Fareed, J., Walenga, J.M. and Emanuele, R.M.: Protamine Neutralization of the Functionality of Heparin and Its Fractions. Thrombosis and Hemorrhagic Diseases, Gozlem Printing and Publishing Co.,Instanbul/Turkey, p. 411, 1986.

Racanelli, A., Breddin, H.K., Krupinski, K. and Fareed, J.: Antagonism of the Antithrombotic Effects of Heparin and Fraxiparine^{R} in a Rat Model of Laser-Induced Thrombosis. Proceedings of the New York Academy of Science, Structure and Activities of Heparin and Related Mucopolysaccharides, 1989.

Racanelli, A., Hoppensteadt, D. and Fareed, J.: In Vitro Protamine Neutralization Profiles of Heparins Differing in Source and Molecular Weight. Seminars in Thrombosis and Hemostasis, 15:(3), 1-4, 1989.

Emanuele, R.M., Racanelli, A. and Fareed, J.: The Pharmacokinetics of Different Heparin Molecular Weight Fractions Using 2 Assay Methods. Therapeutic Drug Monitoring, 10:(2), 153-159, 1988.

Fareed, J., Walenga, J.M., Hoppensteadt, D., Huan, X. and Racanelli, A.: Comparative Study on the In Vitro and In Vivo Activities of Seven Low-Molecular Weight Heparins. Haemostasis 18:suppl.3, pp.3-15, 1988.

Fareed, J., Walenga, J.M., Racanelli, A., Hoppensteadt, D., Huan, X., and Messmore, H.L.: Validity of the Newly Established Low-Molecular-Weight Heparin Standard in Cross-Referencing Low-Molecular-Weight Heparins. Haemostasis, 18:suppl.3, pp.33-47, 1988.

Fareed J., Walenga J.M., Hoppensteadt D., Racanelli A. and Coyne E. Chemical and Biological Heterogeneity in Low Molecular Weight Heparins:Implications for Clinical Use and Standardization, Seminars in Thrombosis and Hemostasis, 15:(3), 135-142, 1989.

Hoppensteadt, D.A., Racanelli A., Walenga J. and Fareed J.: Comparative Antithrombotic and Hemorrhagic Effects of Dermatan Sulfate, Heparan Sulfate and Heparin, Seminars in Thrombosis and Hemostasis, 15:(3), 135-142, 1989.

ABSTRACTS

Racanelli, A., Fareed, J. and Zarudo, J.: Variations in the Neutralization Profiles of Beef Lung and Porcine Mucosal Heparins By Protamine Sulfate. Federation Proceedings, 45: 1986.

Racanelli, A. and Fareed, J.: Differential Neutralization of Unfractionated (UH) and Low Molecular Weight Heparins (LMWHs) By Human Platelet Factor 4. Federation Proceedings 46, 1987.

Racanelli, A., Fareed, J. and Huan, X.: Low Molecular Weight Heparin Induced Bleeding Can Be Neutralized By Protamine. Haemostasis, 18:(Suppl), 163-164, 1988.

Racanelli, A., Breddin, H.K., Krupinski, K. and Fareed, J.: Antagonism of the Antithrombotic Effects of Heparin and Fraxiparine^{R} in a Rat Model of Laser-Induced Thrombosis. Haemostasis, 18 (Suppl), 102, 1988.

Racanelli A. and Fareed J. Comparative Neutralization of the Antithrombotic and Hemorrhagic Effects of Heparin and CY 216D, Thrombosis and Hemostasis, 62 (1), 122, 1989.

Racanelli A., Silber I. and Fareed J. Comparative Time Course Of Pharmacodynamic Activity of Low Molecular Weight Heparin After Protamine Neutralization, Thrombosis and Hemostasis, 62 (1), 305, 1989.

Casu, B., Marchese, L., Naggi, A., Torri, G., Fareed, J., Racanelli, A. and Walenga, J.M.: Influence of the Sulfation Pattern on Certain Biological Properties of Galactosaminoglycans. Thrombosis and Hemostasis 58:(1), p. 374, 1987.

Fareed, J., Walenga, J.M., Hoppensteadt, D., Emanuele, R.M. and Racanelli, A.: Pharmacologic Inequivalence of Low Molecular Weight Heparins. Thrombosis and Hemostasis 58:(1) p. 374, 1987.

Fareed, J., Walenga, J.M., Hoppensteadt, D., Emanuele, R.M. and Racanelli, A.: Biochemical and Pharmacological Inequivalence of Low Molecular Weight Heparins:Lack of Correlation Between Potency and In Vivo Effects. Vth International Meeting of the Danubian League Against Thrombosis and Hemorrhagic Diseases, Erfurt GDR, 1987.

Hoppensteadt, D., Fareed, J., Walenga, J.M., Emanuele, R.M. and Racanelli A.: Different Low Molecular Weight Heparins Are Not Identical in the In Vitro Screening of Potency Adjustment , Thrombosis and Hemostasis, 58:(1) p. 120, 1987.

Huan, X., Fareed, J., Kitsos, G., Kumar, A. and Racanelli, A.: Validity of Animal Models of Bleeding in the Study of the Hemorrhagic Actions of Low Molecular Weight Heparin (LMWH) and Heparin (HEP), Federation Proceedings, 46, 1987.

Svendsen, L., Fareed, J., Racanelli, A. and Hart, R.: Studies on a Synthetic Inhibitor of Thrombin:Application in the Specific Determination of Non-Thrombin Serine Proteases. Federation Proceedings, 45, 1986.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CONTENTS FOR APPENDICES

LIST OF ABBREVIATIONS

1.	APTT	activated partial thromboplastin time
2.	AT III	antithrombin III
3.	AUC	area under the concentration curve
4.	AUMC	area under the moment curve
5.	CBC	complete blood count
6.	Clp	plasma clearance
7.	CPDA	citrate, phosphate, dextrose, adenine
8.	CY 216	a low molecular weight heparin Fraxiparine^R
9.	CY 222	an ultra low molecular weight heparin
10.	D	polymer dispersity
11.	FPA	fibrinopeptide A
12.	FPAGT	fibrinopeptide A generation test
13.	ICU Xa	anti Xa unit (Institut Choay)
14.	IU	international unit
15.	Kabi 2165	a low molecular weight heparin Fragmin^R
16.	LHN-1	a low molecular weight heparin Logiparin^R
17.	mau	maximal activity unit
18.	M_r	molecular weight as determined by gel permeation chromatography (r=molecular radius)
19.	MRT	mean residence time
20.	NHP	normal human plasma
21.	NMP	normal monkey plasma

22. NratP	normal rat plasma
23. NRP	normal rabbit plasma
24. OP/LMWH	a low molecular weight heparin
25. ORG 10172	a low molecular weight heparinoid mixture
26. PAPS	phosphoadenosyl phosphosulfate
27. PCC	prothrombin complex concentrate
28. PK 10169	a low molecular weight heparin Enoxaparin^{R}
29. PT	prothrombin time
30. RT	retention time
31. RVV	Russell's viper venom
32. $t^{1/2}$	biologic half-life
33. t-PA	tissue plasminogen activator
34. USP	United States Pharmacopeia
35. Vd	apparent volume of distribution
36. w/	with

CHAPTER I

REVIEW OF THE LITERATURE

I. HEPARINS

A. History of Heparin

While studying the thromboplastic action of cephalin, a medical student named Jay McClean made the serendipitous finding of a phospholipid anticoagulant in liver extracts (McClean 1916). Soon after, in 1922, a water-soluble glycosaminoglycan complex was discovered by Howell, and named heparin because of its abundance in liver (Howell 1922). Through the work of Scott and Charles (1936), heparin became available in sufficient purity and quantity to make its use in physiological and clinical experiments feasible. In vitro studies by Wessler and Yin, in the early 1970s, demonstrated that thrombosis could be controlled by a block in the coagulation cascade which could lead to an inhibition of thrombin generation and an inhibition of clotting (Wessler 1974; Yin et al. 1973). Clinical trials by Kakaar et al. in 1979 have contributed to the development of low molecular weight heparins as therapeutic agents. Significant advances in the structure activity relationship of low molecular weight heparins by Choay et al. (1983) has lead to the chemical synthesis of a pentasaccharide which contains the minimal binding site of heparin for antithrombin III (AT III).

B. The Biosynthesis of Heparin

Heparin is synthesized as a proteoglycan complex, by connective tissue type mast cells. The synthesis of the glycosaminoglycan is initiated by attachment of a carbohydrate-protein linkage region to serine residues of a specific polypeptide chain (Silbert 1967, Lindahl et al. 1977). After the linkage region is formed the polymer chain of the glycosaminoglycan is assembled by the alternate attachment of N-acetylglucosamine and glucuronic acid. This initial synthesis is followed by postsynthetic modifications which can be divided into four major categories. Initially, glucosamine residues are partially N-deacylated and the exposed amino groups serve as acceptors of SO_4^{-3} ions in a transfer reaction with phosphoadenosyl phosphosulfate (PAPS). Secondly, glucuronic residues are epimerized to iduronic acid moieties. Thirdly, the iduronic acid residues and some glucuronic residues are sulfated at the C-2 position. Fourthly, the glucosamine moieties are ester desulfated to a variable extent at C-3 and C-6 positions (Silbert 1967, Lindahl et al., 1977, Jacobson et al. 1979, Roden and Horowitz 1977). The biosynthesis of heparin does not occur in a random manner, although the regulation of these complex events is yet unclear. Sulfation occurs preferentially in those regions of the chain where the amino sugar residues have been N-deacylated and N-sulfated and where glucuronic residues have been epimerized to l-iduronic acid (Casu 1985). The final product of this synthetic pathway is termed heparin proteoglycan or macromolecular heparin (Yurt et al. 1978; Robinson et al. 1978) and is thought to consist of a core protein of unknown size

with glycosaminoglycan chains of 30,000 to 100,000 daltons, which resembles a bottlebrush (Arnott and Winter 1977).

After heparin is synthesized, it can be modified by three different types of lysosomal enzymes: (1) proteases which can cleave the polypeptide chain, (2) endoglycosidases which can cleave between glucuronic acid and glucosamine residues (Horner 1972; Oosta et al. 1982) and (3) exoglycosidases that remove monosaccharide units from the nonreducing end of the "released" complex carbohydrate species (Neufield et al. 1975). This degradation takes prior to purification of the glycosaminoglycan.

C. Biological Function of Heparin

Heparin is primarily located in the mast cells of a variety of organs, including the liver, lungs, heart, kidneys and intestines. The concomitant occurrence of heparin and histamine in mast cell granules has lead to suggestions that these two biologically active compounds may be stored together as an inactive complex (Unvas 1974). An in vitro complex arising from the ionic binding between the ester sulphate groups of the heparin and the amino groups of the histamine was taken to support this hypothesis (Unvas 1974). Ultrastructural analysis of human lung mast cells demonstrates that the contents of the secretory granules are packaged in a highly ordered crystalline array, suggesting that such complexes occur (Schwartz and Bradford 1986). A unique property of heparin in the mast cells is the high degree of sulfation of heparin which creates an enormous electrostatic charge that can act as a cation exchanger in the granule. In addition, heparin may function

to regulate the osmotic pressure of the granule in the mast cell (Caulfield et al. 1980).

There is no direct evidence that mast cells secrete heparin into the bloodstream. Blood coagulability is generally not effected when mastocytomas of varying degrees of differentiation occur in man, rodents, cattle, cats and dogs, even when large tumors are present (Sagher and Even-Paz 1967).

D. Chemistry of Heparins

Heparin structure consists of a regular and an irregular region. The regular region contains trisulfated disaccharide units (I_{2s}-$A_{ns,6s}$(alpha-1,4-linked L-iduronic acid 2-sulfate)-(D-glucosamine N,6-disulfate)) which are interrupted by other usually less sulfated sequences (Casu 1984). Contained within the regular sequences of the heparin regions is a unique sequence, a pentasaccharide, which binds tightly to antithrombin III (AT III) with a $K_a = 7 \times 10^6\ M^{-1}$ forming an equimolar complex with and enhancing the inhibition of factor Xa by AT III. The pentasaccharide represents the minimal binding sequence of heparin for AT III (Choay et al. 1983).

Approximately 30% of heparin preparations on a weight basis are able to bind to AT III and potentiate the inhibition of the coagulation proteases (Choay and Petitou 1986). As many as 21 different subfractions have been described in heparin, (McDuffie et al. 1975 and Dietrich et al. 1975) however, only 25% to 35% of a given heparin preparation binds tightly to AT III and this high affinity fraction is responsible for 85 to 90 % of the anticoagulant activity of heparin (Lam et al. 1976). Most heparin oligosaccharides which bind to AT III

affinity columns differ in size as well as internal composition which suggests that some residues may be either critical or essential for activity while others may be nonessential for activity (Casu et al. 1981).

Clinically used heparin is heterogenous with respect to structure, molecular size, degree of sulfation and anticoagulant activity. Fragments of heparin have molecular weights which range from 6,000 to more than 30,000 daltons with the largest fraction being between 10,000 and 15,000 daltons (Andersson and Barrowcliffe et al. 1979; Hook et al., 1976). The degree of sulfation contributes to the anticoagulant activity of heparin. A relationship exists between the anticoagulant potency of a heparin and the linear charge density of a series of heparin fractions (Hurst et al. 1979). The anticoagulant activity of fractions with similar molecular weight profiles increases with increasing charge density. However, differences in molecular weight produce larger differences in anticoagulant potency than differences in anionic density (Hurst et al. 1981).

Heparins obtained from various organs and different heparin preparations from the same organ contain differing proportions of regular and irregular sequences which include the linkage region or AT III binding sequence. Typically beef lung heparin contains a larger proportion of the regular trisulfated disaccharide sequences than heparin from porcine mucosal origin. Electrophoresis of heparin in cationic buffers provides further evidence of the heterogeneity of heparin (Casu 1984). In these systems, heparin can be subfractionated into slow moving and fast moving components and preparations from beef

lung usually contain more slow moving species than porcine mucosal heparins.

E. Fractionated and Depolymerized Low Molecular Weight Heparins

Low molecular weight heparin fractions and fragments have been prepared from conventional heparin by several methods. Procedures such as gel filtration, ion exchange chromatography, partition fractionation and affinity chromatography separate the heparin structures based on physiochemical characteristics without cleaving the heparin molecule. Gel filtration has been used to elucidate the molecular weight of the heparin components (Losito 1981). Studies using specific molecular weight components of heparin have shown that anticoagulant activity increases with the size of the molecule and that anti IIa inhibition is more dependant on molecular weight than is anti Xa activity (Andersson et al. 1979). Ion exchange chromatography has been used to separate fractions which have varying anticoagulant activity. Partition fractionation procedures have been used to produce heparins with varying degrees of sulfation which parallel the anticoagulant activity of heparins. Low molecular weight heparin has also been fractionated on affinity columns of substances such as AT III, heparin cofactor II, or thrombin (Choay and Petitou 1986).

Low molecular weight heparin can also be produced by a variety of techniques which cleave the heparin molecule including: heparinase treatment, nitrous acid degradation, perioxadative oxidation followed by either reduction and mild acid hydrolysis or alkaline cleavage fractionation with dilute acid followed by re-N-sulfation of N-desulfated fragments or with chlorosulfonic acid (Casu 1984).

End residues of fragments are typical of the specific method of depolymerization. Heparinase splits glycosidic bonds between N-sulfated glucosamine and iduronic 2-sulfate residues producing fragments terminating with 4,5-unsaturated iduronic acid 2-sulfate at the nonreducing end and N-sulfated glucosamine at the reducing end (Linker et al. 1977). Nitrous acid splits glycosidic bonds between N-sulfated glucosamine and uronic acid, producing fragments with 2,5-anhydromannose at the reducing end (Cifonelli et al. 1976). Periodate oxidation splits the $C\text{-}2$, $C\text{-}3$ bonds of nonsulfated uronic acids, making the glycosidic bonds of modified residues labile to alkali or (when reduced) to acid (Casu 1984). Direct acid hydrolysis preferentially causes N-desulfation (Conrad 1981) while chlorosulfonic acid can be used to depolymerize and resulfate heparin fragments in one step with the end result being a "supersulfated" heparin fragment with approximately one extra sulfate group per disaccharide unit.

Apart from differences in the yield of these fragments from the variety of fragmentation procedures, the method of depolymerization, in theory, may influence the binding sequence to antithrombin III. In principle, nitrous acid could split some glycosidic linkages in the region of the active site for AT III and perioxadative cleavage could conceivably modify the active site for AT III. However, it has been reported the 3-O sulfo group on $A^{*}_{NS\cdot 6S}$ has some stabilizing influence of the corresponding glycosidic linkage (Casu 1981). It has not been determined whether the end group influences the anticoagulant activity of heparin, however, end groups influence the metabolism of the heparin (Linhart personal communication).

Methods which fragment heparin may be preferred over fractionation techniques since the yield of low molecular weight heparin obtained through chromatographic techniques is only about 10 to 15 % with the use of carefully controlled depolymerization techniques nearly 100 % of the sample can be converted to the molecular weight range of choice (Barlow and Petracek 1986).

F. Overview of Hemostasis

Prior to a detailed discussion of the interaction of heparins with the components of the hemostatic system, an overview of the current knowledge on this subject is presented. A schematic diagram of the coagulation cascade is shown in Appendix I.

Blood normally circulates through the vascular system without activation of the coagulation proteins or platelets and without hemorrhage. An injury to a vessel, however, initiates a complex chain of events resulting in the formation of fibrin. Hemostasis is maintained through the interaction of the coagulation and fibrinolytic systems, platelets and the endothelium. The process of coagulation may be described as a waterfall or cascade involving cofactors, enzymes and substrates. Most coagulation enzymes are zymogens containing serine, histidine and aspartic acid in their active sites in addition to containing binding regions to cellular surfaces, phospholipids, cell membranes and connective tissue. The molecular assembly of cofactor, enzyme and substrate is a recurrent theme in blood coagulation designed for maximal efficiency and speed of molecular reactions. Negatively charged surfaces provide the reaction surface in the early part of the

cascade while later phospholipid or platelet membranes provide the surface for reaction in the later part of the cascade.

The initiation of blood coagulation occurs through either the activation of the intrinsic or extrinsic pathway. The intrinsic pathway becomes activated when the coagulation factor XII comes in contact with a foreign surface such as a damaged vessel wall or prosthetic device and becomes activated to factor XIIa. Factor XIIa in the presence of Ca^{+2} converts zymogen factor XI to factor IXa. Factor IXa in the presence of factor VIII and phospholipid activates factor X to factor Xa.

The extrinsic system is involved in response to an injury in the vessel wall and also converts factor X to factor Xa. Components of the extrinsic system include factor VII, a vitamin K dependant enzyme, tissue factor or thromboplastin, a phospholipid which acts as a cofactor in the conversion of factor X to factor Xa by factor VIIa. Once factor Xa is formed by either the extrinsic or intrinsic pathway, it converts prothrombin to thrombin which cleaves fibrinogen to fibrin. Thrombin occupies a central role in hemostatic plug formation and limitation as it acts on multiple substrates including fibrinogen, factor XIII, factor V and factor VIII, platelet membrane glycoprotein V and protein C (Colman et al. 1987). The final two steps of the coagulation are termed the common pathway.

Platelets play an important role in coagulation by releasing substances which stimulate vessel wall constriction and by forming a primary platelet plug at a site of vessel injury. Many of the coagulation factors are attached to the platelet surface where activation of

the coagulation factors takes place. Platelets, upon activation, release substances from their granules which influence blood coagulation. One such substance released from the dense granules is platelet factor 4 which complexes with heparin and neutralizes its activity.

Many plasma proteolytic inhibitors control and limit the extent of coagulation and fibrinolysis. C_1 inhibitor is the major plasma inhibitor of factor XII (Liu et al. 1979), while $alpha_1$ antitrypsin is the major inhibitor of factor XI_a (Ratnoff et al. 1977). AT III is the major inhibitor of factor Xa, XIa and thrombin (Colman et al. 1988). The interaction between AT III and heparin is responsible for most of the anticoagulant effects of heparin.

Fibrinolysis, a physiologic mechanism for limiting clot formation resembles the coagulation cascade in its zymogens, feedback potentiation and inhibition and plasma inhibitors. Endothelial cells release tissue plasminogen activator which convert plasminogen to plasmin. Once plasmin is produced locally on the hemostatic plug, the potential for fibrin degradation exists. An intricate balance exists between the simultaneous forces which either promote or inhibit coagulation and platelet aggregation. The balance between profibrinolytic and antifibrinolytic reactions, in addition to cellular mechanisms may eventually lead to clot reduction (Colman et al. 1987).

G. Mechanism of Action of Heparins

The primary mechanism of the anticoagulant actions of heparin occurs through association of heparin with AT III, an $alpha_2$ globulin with a molecular weight of approximately 64,000 daltons (Abligaard et al. 1967). Heparin forms a stable complex through binding allosteric

lysyl residues on antithrombin III. This results in a conformational change in the protein and allows the critical arginine residue on AT III to be more readily available to interact with the serine active site in a specific activated enzyme. The reactivity between arginine and active serine sites has lead to the hypothesis that heparin-antithrombin III complexes inhibit other serine proteases in addition to thrombin (Rosenberg et al. 1975; Atha et al. 1987). It has been demonstrated in vitro that the heparin-antithrombin III complexes exert their anticoagulant effects by inhibiting factors XIIa, XIa, Xa, IXa and thrombin (Rosenberg et al. 1975). Although most of the in vitro anticoagulant activity of heparin can be related to heparin molecules with high affinity for AT III, antithrombotic effects of heparin have been described which are independent of AT III (Ofosu et al. 1981). These include potentiation of the effects of high affinity heparin by low affinity heparin, binding of heparin to endothelium and the direct inhibition of heparin on several thrombin dependant reactions including factor V and factor VIII activation (Hurst et al. 1983). Heparin also inhibits thrombin through heparin cofactor II, a glycoprotein of 65,600 daltons (Colman et al. 1987). In vitro, the thrombin inhibitory effect has been demonstrated at higher concentrations of heparin that is necessary to inhibit thrombin through AT III (Andrew et al. 1986).

H. In Vitro Effects of Heparins

1. Anticoagulant and Antiprotease Effects

The heterogeneity and polydisperse nature of heparin does not permit the direct measurement of heparin concentration by chemical methods, rather, measurement of heparin activity has been used to

approximate the actual concentration of heparin administered. For many years the anticoagulant activities of heparin have been evaluated using nonspecific global assays such as the USP assay and APTT tests. These assays, however, proved to be insensitive to the newly developed low molecular weight heparins. Newer assays have been developed which are more sensitive to the antiprotease activities of heparin and are useful in the monitoring of low molecular weight heparins. These assays include: serine protease inhibition assays such as anti factor Xa and anti factor IIa methods, serine protease generation tests, fibrinopeptide A generation tests, thrombin generation tests as well as complex combination tests such as the Heptest^{R} which combines enzyme generation, protease inhibition, and clot formation (Fareed et al. 1980).

A molecular weight dependence of the anticoagulant activity of heparin has been demonstrated using the more specific antifactor assays. Denton et al. (1983) and Mardigian and Trillou (1983) have determined that an octadecasaccharide was the minimum length of heparin chain necessary to accelerate the neutralization of thrombin by AT III. Low molecular weight heparins of < 5,600 daltons do not show any significant antithrombin activity. This molecular weight corresponds to the minimum size of heparin to accelerate thrombin inhibition in the presence of AT III (Oosta et al. 1981). Inhibition of factor Xa, however, did not show a similar correlation to molecular weight. Unlike the anti factor IIa effects of heparin. both low and high molecular weight heparin molecules are capable of inhibiting factor Xa. The anti Xa activity of heparin increases with decreasing molecular weight in

both amidolytic and clot based assays (Lane et al. 1978; Andersson et al. 1979; Emanuele 1987). Choay et al. (1980; 1983) and Lindahl and Bjork (1983) determined that the minimal binding site of heparin for AT III was restricted to a pentasaccharide sequence.

At equigravimetric concentrations, unfractionated heparin contains greater anti Xa and anti IIa activity than many low molecular weight heparins (Walenga 1986). When comparing antiprotease activities within a single heparin preparation, one would expect to see approximately equal anti Xa and IIa activity. A greater proportion of anti Xa activity to anti IIa activity is seen however with low molecular weight heparin preparations (Walenga 1986). This difference in relative activity between low molecular weight heparin and unfractionated heparin is due to the fact that low molecular weight heparins contain a greater proportion of oligosaccharides (>50%) which inhibit factor Xa activity. Commercial heparins contain only 5% saccharides with a molecular weight < 4,200 daltons (the minimum weight required to inhibit thrombin).

Some low molecular weight heparins can inhibit thrombosis in vivo while possessing minimal or no anticoagulant activity in vitro (Walenga 1987). New terminology has been introduced which expresses the discrepancy between the in vitro and in vivo activities of these antithrombotic agents. Anticoagulant activity is defined as the ability to inhibit either the activation of prothrombin and/or factor X or the activated enzymes (thrombin and factor Xa respectively). Antithrombotic activity is defined as the in vivo prevention of

thrombosis or the inhibition of the extension of established thrombi (de Prost 1986).

2. Interactions With Platelets

A wide spectrum of in vitro results on the interaction of heparin with platelets has been reported. These include the inhibition of platelet aggregation and release (Han et al. 1974), potentiation both in vitro and in vivo (Thompson et al. 1973; Heptinstal et al. 1977) or no detectable effect (MacFarlane et al. 1975). These differences in experimental results may depend upon the source of heparin or the experimental protocol used in the studies. Heparin blocks the action of thrombin on platelets, while the other activities of platelets may proceed despite the administration of heparin (Salzman et al. 1980). Heparin enhances platelet release induced by ADP, however there is no significant effect of low molecular weight heparins on platelets (Fabris et al. 1983).

Heparin enhances platelet aggregation in citrated platelet rich plasma after induction by various agonists such as ADP and epinephrine. This action is blocked by substances that elevate cAMP and by EDTA but not by inhibitors of the cyclooxygenase pathway (Salzman et al. 1980). A molecular weight dependance of the enhancement of platelet aggregation by heparin has been reported. Fractions of high molecular weight heparin (20,000 daltons) were more reactive with platelets than fractions of low molecular weight heparin (7,000 daltons). Brace et al. (1986) have demonstrated a molecular weight dependance on platelet aggregation using subfractions of a low molecular weight heparin, PK 10169. Preparation of high molecular weight heparin of high and low

affinity were equally active in the induction of platelet aggregation. No correlation between the anticoagulant, antifactor Xa, antifactor IIa activities of the heparins and platelet aggregation was noted.

Heparin does not induce aggregation in washed platelets which suggests that there is not a direct interaction between platelets and heparin (Fabris et al. 1983; Westwick et al. 1986). Binding of heparin to AT III has been reported to inhibit the interaction of heparin with platelets. Shanberge et al. (1986) have demonstrated that platelets bind heparin but not heparin-AT III complexes. Heparin has been shown by O'Brien et al. (1969) and Fabris et al. (1983) to inhibit platelet release induced by collagen in PRP. Heparin may alter the surface charge of platelets and interfere with platelet-collagen interactions.

3. Heparins and Fibrinolysis

Heparin has a dual effect on fibrinolysis in vitro. Levels > 125 μg/ml of this agent inhibited fibrinolysis mediated by human urokinase, human euglobulin fractions and human plasma, while lower heparin concentrations (5 μg/ml) have been shown to enhance fibrinolysis in vitro (Von Kaulla et al. 1958). More recently Paques et al. (1986) and Fears et al. (1988) have demonstrated a concentration dependant stimulation of plasminogenolytic and fibrinogenolytic activity of both urinary and tissue type plasminogen activators in the presence of heparin. In the presence of soluble fibrin, however these effects of heparin on tPA were attenuated (Fears et al. 1988). Heparin also decreased the fibrin specificity of tPA and consequently caused a cleavage of fibrinogen. This may be related to any increased bleeding observed when these agents are used in combination. In

addition, if these influences of heparin and fibrin also occur in vivo, then, in the presence of heparin, the relative fibrin enhancement of tPA will be diminished and the likelihood of systemic activation by tPA is increased (Fears et al. 1988). Markwardt et al. (1977), have shown that heparin induces the release of plasminogen activator using an isolated pig ear preparation.

I. In Vivo Effects of Heparin

1. Studies in Animal Models

The ideal ratio of the antifactor Xa and antifactor IIa activities of heparins needed to achieve optimal antithrombotic effects has not been determined. Antithrombotic effects which are achieved by inhibiting factor Xa alone are limited in their control of various thrombogenic triggers. Better antithrombotic effects are achieved by heparin or heparin-like substances capable of influencing the inactivation and or generation of thrombin. The ability of heparin or a heparinoid to potentiate the inactivation or the generation of thrombin appears to be an important predictor of antithrombotic effect observed in vivo (Buchanan et al. 1984). Heparin fractions with predominately anti factor Xa activities are likely to be weak inhibitors of thrombin generation. Heparin or related substances which exhibit thrombin inhibition are more active against thrombosis as demonstrated in a modified Wessler model than agents with sole inhibitory activity towards factor Xa activity. Although the anti Xa levels of standard heparin and an octasaccharide were similar (0.28-32 U/ml), heparin (10 anti Xa U/kg) inhibited thrombus formation by 90%, while octasaccharide (10 anti Xa U/kg) inhibited thrombus formation by 41%. Dermatan

sulfate (500 μg/kg i.v.), which represents a gravimetric amount 5 times heparin and exhibits no anti Xa activity inhibited thrombus formation by 95%. Also higher doses of octasaccharide which increased anti Xa to higher levels had no further effect on thrombus formation (Buchanan et al. 1984).

Similarly, Walenga et al. (1987) have demonstrated that a pentasaccharide representing the critical sequence required in heparin for binding to AT III, however, devoid of any anti factor IIa activity was capable of inhibiting thrombosis induced by four different thrombogenic challenges. The agents used for the induction of thrombosis in a modified Wessler model included thromboplastin, an activated prothrombin complex concentrate, a nonactivated prothrombin complex concentrate administered simultaneously with Russell's viper venom, and activated factor X. The studies demonstrated that oligosaccharides devoid of antifactor IIa activity but containing high antifactor Xa activity were capable of inhibiting thrombosis induced in rabbit stasis models, however, higher doses of these agents as compared to heparin were required for this effect.

In the treatment of thrombosis, prophylaxis as well as therapeutic intervention to inhibit thrombus extension is important. Low molecular weight heparins (CY 216 and PK 10169) and heparin were shown to be equipotent at doses of 42-62.5 anti Xa U/kg/hr in preventing the accretion of ^{125}I-fibrin onto venous thrombi preformed in rabbit jugular veins (Boneu et al. 1985). Low molecular weight heparins, therefore, could be used for the treatment as well as prophylaxis of thrombosis. The ED_{50} for the antithrombotic effects of low molecular

weight heparins ranged from 40 to 80 μg/kg as compared to an ED_{50} of approximately 30 μg/kg for unfractionated heparin as demonstrated in a stasis thrombosis model (Fareed et al. 1988).

Many theories have been proposed to explain the potentiation of the actions of high affinity heparin oligosaccharides by low affinity heparin in vivo. These include: the binding of low affinity heparin to antiheparin proteins in plasma, interactions of the low affinity heparin with vascular endothelial cells or providing an alternative substrate to endoglycosidase enzymes located in platelets (Barrowcliffe et al. 1984). Two heparin fractions: one with high affinity to AT III and one with low affinity to AT III were compared with unfractionated heparin in both in vitro assays and in an experimental model of stasis thrombosis (Merton et al. 1984). Although the in vitro activity of high affinity heparin was twice that of unfractionated heparin, the high affinity heparin had less antithrombotic activity than the unfractionated heparin. The low affinity heparin was virtually inactive, both in vitro and in vivo. A mixture of 30 μg/kg of high affinity heparin and 50 μg/kg of low affinity heparin given intravenously was as effective as unfractionated heparin (80 μg/kg i.v.). These studies demonstrate that low affinity heparin potentiates the actions of high affinity heparin in vivo.

With the development of low molecular weight heparins, it became possible to dissociate the antithrombotic and hemorrhagic effects of these agents, thus improving their therapeutic potential (Cade et al. 1984). Low molecular weight heparins produce less bleeding than unfractionated heparin, however no strong relationship exists between

blood loss and the effects on antifactor Xa levels, APTT, thrombin clotting time as measured ex vivo (Cade et al. 1984).

Heparin fractions of low molecular weight with high antifactor Xa activity/APTT activity ratios exhibit antithrombotic activity and are associated with a reduced incidence of bleeding (Mattsson et al. 1985). Doutremepuich et al. (1986) have demonstrated that a low molecular weight heparin, CY 216, had lower hemorrhagic effects than heparin as measured in a rabbit ear blood loss model. At doses of heparin (1408 anti-factor Xa U/kg) and CY 216 (1600 anti-factor Xa U/kg) with similar anti Xa activities, the blood loss values for heparin were significantly greater ($p<.05$) than for CY 216. Studies on the time course of antiprotease, antithrombotic and bleeding effects of heparins in rats by Hobblelen et al. (1985) have shown that the time response curves for the antithrombotic effects of heparins parallels those of the antifactor Xa activity whereas the time response curves for bleeding were more related to the effects on thrombin.

2. Pharmacokinetics/Pharmacodynamics of Heparins

The pharmacokinetics of heparin are nonlinear, dose dependant, time dependant and assay dependant (Cocchetto and Bjornsson 1984). These properties contribute to the variations seen in anticoagulation for patients given a certain dose of heparin. Pharmacokinetic parameters depend upon the assay used to determine heparin activity. APTT activity has been reported to disappear faster than anti Xa activity shortly after drug administration (de Swaart et al. 1982). Four fold variation in the $t_{1/2}$ of a low molecular weight heparin was seen by Dawes et al. (1986) and was dependant upon whether concentration was

measured directly or anti Xa, or anti IIa methods were used (Dawes et al. 1986).

The dose and route of administration of heparin depends upon the clinical indication. Heparin is not effective by oral administration and is given either by deep subcutaneous injection, intermittent intravenous injection or by intravenous infusion. The dose of heparin is adjusted according to the patients coagulation test results. The APTT should be elevated to approximately 1.5 to 2 times the control value (Salzman 1975).

For subcutaneous administration of heparin, 5,000 U of heparin is given initially by intravenous injection and followed by 10,000 to 20,000 U given subcutaneously. Then 8,000 to 10,000 U of a concentrated solution are given every 8 hours subcutaneously. For intermittent intravenous injection of heparin, 5,000 to 10,000 U of heparin is administered every 4 to 6 hours. For continuous intravenous infusion, an initial injection of 5,000 U of heparin then an additional 15,000 to 35,000 U are administered over a period of 24 hours. Patients undergoing open heart surgery should receive an initial dose of 150 U/kg of heparin. Frequently a dose of 300 U/kg is used for procedures estimated to last less than 60 minutes; or 400 U/kg for those estimated to last longer than 60 minutes. Low dose heparin given prior to and after surgery has been shown to reduce the incidence of postoperative deep venous thrombosis. The most widely used dose is 5,000 U, 2 hours before surgery and 5,000 U every 8 to 12 hours after for 7 days until the patient is fully ambulatory (Physician's Desk Reference 1989).

a. Absorption of Heparins

Heparin is not absorbed to any great extent by gastrointestinal mucosa, due to its negatively charged high molecular weight components. Oral absorption of heparin is variable because of its uncertain absorption from the G.I. tract and its instability in digestive juices, in addition, intestinal flora has also been noted to degrade heparin (Brufani et al. 1982). The N-sulfate groups of heparin are easily hydrolyzed in acidic medium which may result in a substantial loss of anticoagulant activity. While heparins of molecular weight >6000 daltons do not cross the intestinal barrier, low molecular weight heparins (mean molecular weight approximately 4500 daltons) are absorbed from the gastrointestinal tract at very high doses (60 mg/kg).

Heparins have been complexed with bile salts, (Ziv et al. 1983; Guarini et al. 1985) surface active agents (Stanzani et al. 1981) and encapsulated within liposomes (Ueno et al. 1982) in order to facilitate gastrointestinal absorption in experimental animals. The lower molecular weight of the newly developed heparins has facilitated the study of percutaneously applied low molecular weight heparins (Rodemer et al. 1986). Absorption of low molecular heparin in healthy subjects was meager at the doses studied (100-250 anti Xa units). It is conceivable however, that with better carrier substances absorption by percutaneous route could be improved. The rate of absorption depends upon the injection site and has been shown to exhibit intrastrain or interstrain variations in experimental animals. Variations in the subcutaneous absorption between two strains of mice have been reported

by Sue (1979). Variability in the subcutaneous absorption of unfractionated heparin has been reported in man, although these variations decreased with decreasing molelcular weight, this variability could not be correlated to any physical parameter such as age, height or weight. A barrier to absorption exists which discriminates against larger molecules such that the 6 fold variation in AUC values between subjects observed with unfractionated heparin was reduced to 1.5 fold for low molecular weight heparin CY 222 (Dawes et al. 1986).

It was generally assumed that the activity of heparins in vivo resembled their in vitro anticoagulant and antiprotease activity profiles. However, differences between these two situations exist. Investigators have shown that heparin is not inert as it circulates in the plasma. Endogenous heparin can bind to the glycocalyx and be phagocytosed (Barzu et al. 1985). While the uptake of heparin is well documented, there is only indirect evidence for its eventual release in an undegraded form (Mahadoo and Jacques 1979). Extended anticoagulant responses observed with more indirect routes of administration could be related to either a slow absorption from the site of administration or a slow release of heparin which is sequestered by the reticuloendothelial system. The duration of anticoagulant response (parentheses indicate half life) increases with the route that allows for greater cellular uptake of heparin as follows: intrapulmonary (14 days) > subcutaneous (6-8 hrs) > intramuscular (4-6 hrs) > intravenous (2-4 hrs), (Mahadoo et al. 1979).

It has been postulated by Mahadoo et al. (1979), that a cellular pool exists which modulates the level of heparin in the circulation and

may be related to endothelial cell binding of heparin. An increase in the size of the cellular pool causes a decreased availability of heparin to the circulating blood. This pool may act as a storage depot from which stored heparin is gradually released to the circulation. The degree of cellular uptake varies between the different sites of administration as follows: lung >peritoneal cavity > skin > muscle corresponds to the cell population of reticuloendothelial cells at the site of administration (Mahadoo and Jaques 1979). Heparin is rapidly absorbed and metabolized after parenteral administration. Although after intrapulmonary administration 90% of the drug is absorbed in 24 hours while the anticoagulant response lasts for more than one week. This suggests that heparin is sequestered and released into the circulation (Jaques et al. 1976).

Low molecular weight heparin is absorbed from subcutaneous sites to a greater extent than standard heparin. After subcutaneous administration of 7500 anti Xa U, the anti Xa activity of heparin is absorbed to a lesser degree than CY 216 (Harenberg et al. 1986). Twelve hours after the administration of 5000 anti Xa IU of heparin and a low molecular weight heparin, higher anti Xa levels were seen for the low molelcular weight heparin than the unfractionated heparin ever produced (Bergvist et al. 1983).

b. Endogenous Distribution of Heparins

The polycomponent nature of heparin makes the study of its distribution within the body difficult. Since heparin contains many different molecular species with varying structures and degree of sulfation, it is not possible to uniformly radiolabel heparins.

However, radiolabelling studies have been performed and have shown that after intravenous administration, the anticoagulant activity of heparin disappears exponentially with a slightly faster initial disappearance which may be representative of endothelial uptake (Jacques 1979).

Once heparin enters the vascular compartment it binds to many plasma proteins including AT III, albumin, globulins, fibrinogen, fibronectin, lipoproteins, and platelet factor 4 (Cocchetto and Bjornsson 1984). Heparin is also taken up by vascular endothelium and tissue macrophages (Mahadoo 1978).

The distribution of ^{35}S heparin fragments (1 mg/kg i.v.) was studied in rats by Larsen et al. (1984). Low levels of radioactivity were present in all organs and tissues with an overall recovery of radioactivity of 94.4 %. 78% of organ bound radioactivity was associated with the liver, kidneys and intestines. The highest disposition of radioactivity was found in the liver while the highest specific activity (dose/gram) was associated with the kidneys. The organ distribution of heparin and low molecular heparin is similar. Both drugs exhibit a selective distribution in various organs especially those endowed with reticuloendothelial tissue (Danishefsky and Eiber 1959).

c. Half Life of Heparins

The $t_{1/2}$ of low molecular weight heparin is usually 2 to 3 times the $t_{1/2}$ of unfractionated heparin and is dependant on the dose, route of administration and assay used to detect anticoagulant activity (Lockner et al. 1986; Fareed et al. 1986). The $t_{1/2}$ of heparin increases with increasing dose (Estes et al. 1969). The immediate

mechanism underlying the dose dependant kinetics of heparin, in rats, appears to be a decrease in total clearance and an increase in apparent V_d with increasing dose (Bjornsson et al. 1981).

In primates, the $t_{1/2}$ of unfractionated heparin (250 μg/kg i.v.) as measured by the Heptest^R is approximately 30 minutes while the $t_{1/2}$ of low molecular weight heparin CY 216 (1 mg/kg i.v.) is 1 hour (Emanuele 1987). After subcutaneous administration, the half life of CY 216 is longer than for unfractionated heparin. The $t_{1/2}$ of CY 216 (1 mg/kg s.c.) is reported to be 144 minutes while the $t_{1/2}$ of unfractionated heparin (1 mg/kg sc) is 94 minutes as determined by the Heptest^R (Emanuele 1987).

Binding to plasma proteins such as the AT III increases the $t_{1/2}$ of heparin. AT III heparin complexes disappear more slowly from the circulation than heparin which is not bound to AT III. The $t_{1/2}$ of a hexadecasaccharride complexed to AT III and a dodecasaccharide complexed to AT III is 30 times longer than that of heparin and 10 times longer than AT III-heparin complexes in rabbits (Mattsson et al. 1985). The encapsulation of heparin in liposomes also increases the $t_{1/2}$ of intravenously administered heparin three times in rats (Kim et al. 1986).

In addition to having an increased $t_{1/2}$ which permits once daily injections of low molecular weight heparins, these drugs also exhibit increased bioavailability as compared to heparin after subcutaneous injection (Palm and Mattson 1987). The low molecular weight heparin, Fragmin^R, is 86% bioavailable after subcutaneous administration as compared to 10% for unfractionated heparin (Lockner et al. 1986).

d. Metabolism of Heparins

In 1945, Monkhouse and Dickie observed that heparin had a longer survival time in the circulation after blocking the reticuloendothelial system (RES) of rabbits with thorotrast (Monkhouse and Dickie 1954). This suggests that heparin is eliminated via the RES. Heparin is desulfated by N-sulfatases and sulfamidases of the liver and subsequently depolymerized into oligosaccharides (Cocchetto and Bjornsson 1984). Other investigators have shown that cells of the RES can phagocytose heparin and store it for varying lengths of time (Oh et al. 1973). Endothelial cells selectively bind those chains of heparin which have a higher molecular weight, greater charge density and greater anticoagulant activity. Fragments released back into the circulation exhibit low anti IIa activity but retain their anti Xa activity (Barzu et al. 1987). Heparin structural determinants for the anti Xa activity are more resistant to enzymatic attack in comparison to those responsible for the antithrombin activity (Mastacchi and Barbati 1987). Endocytosis may be followed by depolymerization of internalized heparin by lysosomal enzymes.

Heparins are differentially metabolized by kidneys. Low molecular weight heparin is a poorer substrate for renal lysosomal inactivators than unfractionated heparin (Mastacchi and Barbanti 1987).

e. Clearance of Heparins

The liver and kidneys play a fundamental role in the elimination of heparin. Heparin elimination is prolonged in rabbits with renal dysfunction and with reticuloendothelial systems that are blocked by

various agents (Palm and Mattson 1987). In humans, cirrhosis of the liver leads to an increase in the half life of heparin (Teien 1977). Severe impairment of renal function does not alter the half life of standard heparin but prolongs the $t_{1/2}$ of low molecular weight heparin (Goudable et al. 1986). Clearance of heparin activity may result from a combination of saturable and a nonsaturable mechanisms (De Swart 1982). The saturable mechanism may be related to heparin binding to the endothelium and reticuloendothelial cells (Dawes and Pepper 1979) while nonsaturable mechanisms may be related to renal elimination of heparin (Piper 1947).

Only small amounts of heparin are excreted unchanged in the urine which suggests that circulating metabolites of heparin exist in plasma. In addition, the extent of urinary excretion of heparin is dependant upon the molecular weight of heparin. Low molecular weight heparin is cleared more readily by the kidneys. By 5 hours, in rats, 54% of injected heparin, 83% of hexasaccharides and 89% of disaccharides are present in the urine (Larsen et al. 1986). The mechanism through which heparin and low molecular weight heparin are cleared by the kidneys differs. Standard heparin is cleared through by an active and saturable cellular mechanism while low molecular weight heparin is cleared by renal filtration (Goudable et al. 1986).

Boneu et al. (1987) studied the disappearance of ^{125}I heparin in rabbits and found a dose dependant increase in the biologic $t_{1/2}$ of heparin due to a decrease in the total clearance of the anticoagulant. Saturable mechanisms of elimination predominate at low therapeutic doses of heparin, (<100 antifactor Xa U/kg) while nonsaturable

mechanisms predominate at higher doses (Boneu et al. 1987). However, saturable mechanisms contribute little to the disappearance of low molecular weight heparins. These agents are cleared primarily by nonsaturable routes such as renal excretion. Low molecular weight heparins also exhibit dose dependant clearances. Longer biological effects have been observed for low molecular weight heparin than for heparin at low doses. However, at higher doses, low molecular weight heparins are cleared faster than standard heparin (Boneu et al. 1987; Samama et al. 1989).

Factors such as affinity to AT III, and molecular weight influence the disappearance rates of heparin. Heparin molecules with varying degrees of affinity to AT III are cleared at different rates. Equigravimetric amounts of ^{125}I-low affinity heparin disappeared 3 times faster than ^{125}I-standard heparin (de Swart 1984). AT III heparin complexes disappear more rapidly from the plasma than free AT III. ^{125} I AT III disappears according to a double exponential curve with a half life in the second phase of 56.8 in the absence of heparin and 33.7 hours in the presence of heparin (de Swart et al. 1984). Molecular weight also influences the disappearance rate of heparins. CY 222 an ultra low molecular weight heparin (mean M_r of 2,500 daltons) was cleared faster than CY 216 with a mean M_r of 4,500 daltons (Dawes et al. 1986).

3. Interactions With Platelets

Clinical complications of heparin therapy involving platelets include bleeding and thrombocytopenia. Two thrombocytopenic syndromes have been described in patients receiving heparin. The first involves

a transient, minor reduction in platelet count which occurs shortly after intravenous injection. In the second case, a more severe thrombocytopenia occurs 5 to 9 days after the initiation of heparin therapy. Approximately 5% of all patients on heparin therapy may develop this severe type of thrombocytopenia which may be associated with arterial thrombosis, stroke, heart attack or death (Kelton et al. 1986).

Low molecular weight heparins exhibit reduced platelet reactivity, and have been used in clinical situations where it was desirable to minimize any platelets effects which may be induced by the unfractionated heparin (Horellou et al. 1984). Low molecular weight heparins have been useful in the treatment of heparin induced thrombocytopenia. Appropriate laboratory tests, however, should be performed before low molecular weight heparin is administered to patients who are suffering from thrombocytopenia in order to test for cross reactivity between heparin antibodies and low molecular weight heparins (Messmore et al. 1983).

4. Heparin and Fibrinolysis

Many investigators have reported that heparin and related substances enhance fibrinolytic activity in both animal models and human volunteers (Fareed et al. 1985; Arnesson et al. 1987; Paques et al. 1986). Increased fibrinolytic activity after the administration of heparin may depend upon molecular weight and degree of sulfation of the heparin, enzyme activation or induction of tPA synthesis. Vinazzer et al. (1982) determined that the activation of fibrinolysis partly depends on the molecular weight and degree of sulfation of the heparin. In a rabbit model of thrombolysis, low molecular weight heparins CY 216

and CY 222 were shown to have a more pronounced potentiation of the effects of rtPA (recombinant tissue plasminogen activator) and scUPA (single chain urokinase plasminogen activator) than with unfractionated heparin (Stassen et al. 1987). Another possible mechanism for increased fibrinolytic activity in the presence of heparin involves the activation of factor XII and kallikrein (Fareed et al. 1985). Recently, it has been shown that heparin induces the synthesis of tPA in human fibroblasts in the presence of an endothelial cell growth factor (Rappaport et al. 1986).

Vairel et al. (1983) have demonstrated a decrease in antithrombotic activity of heparin in a rabbit model after administration of a fibrinolytic inhibitor, epsilon amino caproic acid. This suggests that some of the antithrombotic activity of heparin may be related to fibrinolytic effects induced by heparin.

Low dose heparin (5000 IU) in women undergoing surgery for mammary hyperplasia has been shown to increase tPA antigen before and after venous stasis, with no differences in plasminogen activator inhibitor activity between groups. This suggests that the increase in activity may due to an increase in tPA synthesis in the endothelium (Arnesen et al. 1987).

5. Lipolytic Effects of Heparin

Besides inhibiting the coagulation process, administration of heparin has a marked effect on the metabolism of plasma lipids (Persson et al. 1985). Heparin releases two forms of lipoprotein lipase into the circulation upon <u>in vivo</u> administration. One form of lipoprotein lipase is released from the liver and the release of this lipase is

resistant to protamine neutralization. The release of the other lipoprotein lipase is inhibited by protamine and catalyzes the hydrolysis of plasma triglycerides and induces rapid lipolysis in the bloodstream (Krauss et al. 1974). This leads to a substantial elevation in the plasma level of free fatty acids and partial glycerides (Persson et al. 1985; Nilsson-Ehle et al. 1980). The use of low molecular weight heparins as antithrombotic agents is associated with lower plasma lipolytic activity than with standard heparin (Persson et al. 1987). Subcutaneous administration of 5000 anti Xa units resulted in significantly higher anti factor Xa activity but a lower release of lipoprotein lipase and hepatic lipase than heparin.

6. Toxicity of Heparins

Acute, subacute and chronic toxicological studies of a low molecular weight heparin were performed by Borelli and Bertolli (1986) in rats. 10 mg/kg of heparin presented effects similar to those shown by the low molecular weight heparin (20 mg/kg). A low molecular weight heparin (OP/LMWH) elicited no evidence of teratogenicity when administered by subcutaneous route during the period of organogenesis to pregnant rats at doses up to 10 mg/kg/day. In reproductive studies there were no effects on conception or pregnancy in male or female rats at dosages up to 10 mg/kg/day, by subcutaneous route (Bertoli and Borelli 1986).

Adverse effects associated with the therapeutic use of heparin in man can be categorized into three groups. These are complications resulting from acute heparin administration, chronic heparin therapy and those associated with heparin neutralization. Acute complications

associated with heparin therapy include anaphylaxis, transient thrombocytopenia, arterial embolism and the most frequent complication of heparin therapy, hemorrhage (Gervin 1975).

With the development of purified forms of heparin, anaphylaxis has become a rare complication of heparin therapy. Frequent anaphylactic reactions to heparin, noted during the early period of heparin development, were a result of allergic responses to heparin contaminants rather than to the drug itself. Highly purified forms of heparin have decreased the incidence of anaphylaxis which now occurs in less than 1 % of all persons receiving heparin. However, mild bronchoconstriction, lacrimation, urticaria and rhinitis may occur in approximately 5 per cent of patients (Chernoff 1950).

Thrombocytopenia may occur four to seven days after the initiation of therapy and may be complicated by significant bleeding. Arterial emboli have been reported in a limited number of occurrences, 7 to 15 days after the onset of subcutaneously or intramuscularly administered heparin therapy. The emboli are composed primarily of platelet aggregates in a fibrin matrix. These occur only in the arterial system and may result from heparin induced platelet aggregation (Gervin 1975).

The most frequent complication of heparin therapy is hemorrhage with the incidence of hemorrhage during heparinization being from 8 to 33%. A wide spectrum and severity of hemorrhage during heparin therapy may occur and may vary from mild mucosal oozing to massive intracranial, intrathoracic, gastrointestinal or genitourinary bleeding (Gervin 1975). Patients with low body weight or small stature manifest a

greater prolongation of *in vitro* clotting times than persons with an average size and weight (Gervin 1975). However, there is no correlation between heparin dosage, prolongation of *in vitro* coagulation studies and hemorrhage. Elderly women, however, may have a 50% greater incidence of bleeding during heparinization than the population at large.

Recent attention has been directed to the intraoperative use of small doses of heparin. The treatment of patients with small doses of subcutaneously administered heparin is called the minidose regimen for heparinization and consists of subcutaneous administration of 5,000 to 10,000 IU prior to and at given intervals after surgical procedures (Gervin 1975).

II. PROTAMINE

A. History of Protamine

Friedrick Miescher began investigations of the cell nucleus in 1874 which led to the identification of a nitrogenous base bound to nuclear material of Rhine salmon sperm heads and he named this substance protamine (Miescher 1874). In 1928, Kossel classified protamines into 3 groups according to the number and kinds of amino acids each contained. Monoprotamines contain only arginine as their basic amino acid. Clupeine, salmine and iridine are examples of monoprotamines. Diprotamines contain two kinds of basic amino acids: arginine plus either lysine or histidine. Cyprinine, crenilabrine and barbine are reported to belong to the group containing histidine and arginine. Triprotamines contain all three basic amino acids and include protamines such as sturine (Kossel 1928). Among proteins studied at that

time, protamines possessed the simplest amino acid compositions and Meischer and his colleagues established the fundamentals of protein chemistry using protamines as prototypes (Horrow 1985).

In 1900, Thompson demonstrated that protamine possessed slight anticoagulant activity and speculated that if protamine was complexed to heparin, the activity of heparin may be prolonged in a way analogous to the way protamine prolongs the activity of insulin. However, the complexation of protamine with heparin has found other therapeutic uses since that time when Thompson initially speculated about the use of protamine with heparin. In 1938, Crafoord stated that it was not advisable to administer heparin earlier than 4 hours after finishing an operation because there may be difficulty in controlling hemorrhage in heparinized patients during the operation (Jorpes et al. 1939). When patients are treated with heparin it is sometimes desirable to neutralize its activity in order to control any bleeding which may occur as a result of its administration. Chargraff and Olson, in 1937, demonstrated that protamine may be used to neutralize the anticoagulant action of heparin and this use of protamine would allow the interruption of heparin activity at any time (Chargaff and Olson, 1937).

B. Biosynthesis of Protamine

Protamine is synthesized in the cytoplasm on ribosomes which are present in high concentration during the period of rapid protamine synthesis (Gatewood 1989). These ribosomes incorporate arginine very rapidly. Lysine incorporation on these ribosomes is very slow suggesting that these structures are principally engaged in protamine synthesis (Ling et al. 1969). Protamine appears in the nuclei at a

late stage of spermatogenesis and this newly synthesized protamine progressively replaces histones in combination with DNA. When protamine synthesis begins, histone synthesis declines and eventually ceases (Ingles et al. 1966; Ling et al. 1969). Although histones are structurally conserved, protamines differ considerably from one species to another (Gusse et al. 1983). The newly synthesized protamine is phosphorylated by adenosine triphosphate in the cytoplasm. This phosphorylation occurs at the hydroxyl groups of serine residues, considerably reduces the net charge of protamine and may alter its interaction with DNA (Marushige et al. 1969).

C. Biological Function of Protamines

Relative amounts of the protamine components (C_I, C_{II}, and C_{III}) present in spermatid nuclei vary during the different stages of spermatogenesis. Relative amounts of C_I decrease, while C_{III} increase during testis maturation. This is probably as a result of differing rates of synthesis of each component. The independent synthesis of the individual protamine components suggests that, in spite of their similarities, individual protamines may have specific functions (Ling et al. 1971).

There are two probable functions of protamine in the formation of sperm cells: (a) the clustering of DNA into the sperm head and (b) the total repression of gene expression in the totally differentiated sperm cell. It is possible that packing is the primary function of protamine and that repression of gene expression occurs only as a consequence of the packaging of DNA which makes the genome unavailable for transcription (Ling et al. 1971). Sequence-specific packaging

would suggest distinct structural and functional roles for the nucleohistone and nucleoprotamine in late spermatogenesis or early development or both. Human sperm DNA is packaged into nucleohistone and nucleoprotamine in a sequence specific manner (Gatewood et al. 1988).

In a pure system, using labelled thrombin and AT III, protamine inhibits the inactivation of thrombin (Cobel-Geard and Hassouna 1983). In clotting assays, protamine sulfate has an inhibitory effect on thrombin in the conversion of fibrinogen to fibrin. This inhibition is concentration dependent, partial and reversible. It is postulated that the mode of neutralization of alpha thrombin by protamine is similar to the mechanism by which thrombin is inhibited by AT III with its natural substrate fibrinogen (Cobel-Geard and Hassouna 1983).

D. Chemistry of Protamine

Protamines are a mixture of simple basic proteins containing several components which are similar in structure and amino acid content. Protamines contain mostly arginine although alanine, serine, proline, valine, glycine, isoleucine and threonine are present (Ando et al. 1973). These amino acids have been isolated from the sperm of several mammals, including the boar, ram, rat, guinea pig, rabbit, mouse and human (Tobita et al. 1982). Protamines can also be prepared from various types of fish including herring, salmon and trout.

The molecular weight of various protamines have been determined by a number of chemical methods and found to be between 4500 and 5000 daltons (Ling et al. 1971). Therefore, protamines contain approximately 30 amino acid residues. Fractionation techniques such as countercurrent distribution and chromatography on paper, alumina or ion

exchange columns have revealed the presence of more than one component in each protamine examined (Ling et al. 1971). Heterogeneity is an inherent part of protamines. Differences in amino acid composition are often found among specimens of protamines supposed to have been prepared from the same species. The discrepancy most often occurs in the fractions of lower molecular weight. Deviations of this kind may be accounted for by differences in the maturity and freshness of the starting materials and method of isolation and purification (Ando et al. 1973).

Commercially available protamine is currently prepared from salmon milt, consisting of secretion-laden male gonads. The ground and filtered milt is heated with alcohol (defatted), precipitated with alcohol and sodium chloride, then heated, filtered and depyrogenated. The dried powder, prepared by various manufacturers is packaged and marketed as either powder (Upjohn Company, Kalamoazoo, MI; Institut Choay, Paris, France) or solution (Eli Lilly and Company, Indianapolis, IN), (Horrow 1985).

E. Toxicity of Protamine

Toxicity associated with the administration of protamine sulfate includes: cellular derangements, complement activation, adverse hemodynamic reactions and anaphylaxis. High doses of protamine sulfate can cause mast cell and basophil degranulation with subsequent release of histamine (Frater et al. 1984). Protamine also can cause platelet agglutination and formation of platelet aggregates. Positively charged polymers such as polybrene and protamine could adhere to negatively charged platelet membrane and induce platelet aggregation by forming

bridges between adjacent platelets in a Ca^{+2} independent interaction (Eika 1972).

Protamine administration in experimental animals causes a mild, transient granulocytopenia and thrombocytopenia (Al-Mondhiry et al. 1985;Eika et al. 1971). Protamine is toxic to rats, guinea pigs and mice with an intravenous LD_{50} of approximately 100 mg/kg.

Injection of protamine into rabbits causes a rapid transient fall in platelet number with pulmonary symptoms such as transient coughing, increased respiration and cyanosis (Horrow 1985). Protamine also precipitates fibrinogen in rabbits (Eika and Godal 1971; Gans et al. 1966). Cardiovascular changes also occur including: hypotension, decrease in systemic vascular resistance, an increase in peripheral vascular resistance and increase in cardiac index occur after protamine sulfate administration (Jaques et al. 1949; Conzen et al. 1989).

Recently it has been demonstrated that protamine inhibits carboxypeptidase N, the inactivator of anaphylatoxins and kinins. Heparin competes with carboxypeptidase N for protamine. At high concentrations of heparin (18 U/ml) it reverses the inhibition by protamine. Thus by blocking the inactivation of the mediators released in shock, protamine inhibition of carboxypeptidase N my be partially responsible for the catastrophic reaction which can occur in some patients (Tan et al. 1989).

Low dose protamine pretreatment attenuates the adverse effects of intravenously administered protamine which is used to reverse heparin anticoagulation in clinical settings. Pretreatment with protamine compared with saline solution prevented the hypotension

(+6vs. -16 mmHg $p.< 0.01$) observed with protamine reversal of heparin. Protamine pretreatment lessened the thrombocytopenia found during reversal as compared with saline pretreatment (Wakefield et al. 1986).

Although rare, major reactions to protamine which simulate anaphylaxis occur and have been associated with an allergic reaction to fish. Neutral protamine insulin contains protamine and it has been shown that the incidence of major protamine reaction was 27 % (4/15) in the NPH insulin dependant diabetics vs. 0.5% (3/636) in those with no history of NPH insulin use (Stewart et al. 1984).

Although protamine has been shown to have in vitro anticoagulant effects, overdoses of protamine of as much as 800 mg/70 kg had minimal effects on the coagulation mechanisms of both patients and volunteers. These effects consisted of a slight increase in the Lee-White coagulation time without a concomitant increase in the APTT (Ellison et al. 1971).

F. Other Agents Which Neutralize Heparin Effects

In addition to protamine, several other agents interact with heparin to neutralize its activity, however, protamine is used most widely because it the least toxic of the other antagonists. Polybrene (hexadimethrine bromide) a synthetic heparin antagonist is used in clinical laboratories to neutralize the anticoagulant effects of heparin. In dogs, polybrene has been associated with reduced cardiac output and increased pulmonary vascular resistance. These changes are greater than those demonstrated with the use of protamine. Polybrene has been demonstrated to inhibit factor XII activation (Castenda et al. 1967).

Conley, in 1948, observed that patients with thrombocytopenia exhibited an increased sensitivity to heparin and suggested that platelets could antagonize the anticoagulant effect of heparin. Human platelets contain a heparin-neutralizing protein known as platelet factor 4 (PF4) which is located with other proteins in the alpha granules of platelets and released during platelet aggregation (Dawes et al. 1982).

Protamine is superior to histones in neutralizing heparin. The antiheparin potency of different histone fractions shows differences between fractions (lysine rich histone > crude histone > arginine-rich histone). Poly-DL-lysine is shown to have a wider range of neutralization than protamine (Fabian and Aronson 1980).

Histidine rich glycoprotein also binds heparin and interferes with its interaction with AT III resulting in a neutralization of its anticoagulant activity. In purified systems, histidine rich glycoprotein and high affinity heparin react with apparent 1:1 stoichiometry to form a dissociation constant of 7 nM compared to a dissociation constant of 65 to 200 nM for AT III-heparin complexes (Lijnen et al. 1983; Fabian and Aronson 1980). Another physiological heparin-neutralizing substance is S protein, a major inhibitor of the assembly of the membrane attack complex of complement. S protein, also known as vitronectin, effectively neutralized oligosaccharides of M_r 2,400-7,200. This is unlike the two other physiological inhibitors of heparin, histidine rich glycoprotein and platelet factor 4. In addition, S protein/vitronectin neutralized the anti factor Xa activity

of a synthetic pentasaccharide comprising the antithrombin III binding sequence of heparin (Lane et al. 1987).

III. PROTAMINE-HEPARIN-INTERACTIONS

A. Molecular Aspects of Heparin-Protamine Interactions

Many factors influence the interaction of heparin with protamine which include: charge type, charge density and individual molecular weight of a given heparin or protamine. In view of the opposite surface charge of protamine, the neutralization of heparin activity is assumed to result from intermolecular charge neutralization (Schiele and Heuck 1986). In general, heparin which has high anticoagulant activity also possesses high charge density. In addition stronger polycation binding is observed as the charge density of the heparin is increased. Degree of neutralization of heparin by protamine depends upon the source of heparin (Lowary et al. 1971). Beef lung heparin requires more protamine sulfate than porcine mucosal heparin for neutralization to occur (Racanelli et al. 1989). Each mg of protamine sulfate neutralizes approximately 90 USP Units of heparin of beef lung origin or 115 USP Units of heparin derived from intestinal mucosa (The Upjohn Company, Kalamazoo Michigan, package insert).

Poly-L-lysine has a greater positive charge than protamine and binds to heparin more strongly than does protamine. In addition poly-l-lysine neutralizes heparin over a larger range of concentrations than protamine. The binding strengths of both complexes decrease as the degree of sulfation of the heparin participating in the complex is reduced. Partial N-desulfation of heparin decreases the proportion of binding sites with high affinity for AT III. A complex of partially N-

desulfated heparin and protamine binds more weakly than corresponding heparin-antithrombin III complexes (Jones et al. 1986).

Dawes and Pepper (1982) have shown that the degree of sulfation, position of sulfate residues and molecular weight are important determinants of heparin affinity for protamine. In addition, protamine binds to chondroitin-4-sulfate with greater affinity than chondroitin-6-sulfate. These two chondroitin sulfates differ only by the position of sulfate substitution. Higher molecular weight glycosaminoglycans bind with lower affinity to protamine than the lower molecular weight glycosaminoglycans (Dawes and Pepper 1982).

Whether protamine binds to heparin to form an equimolar complex or a ternary complex has not yet been determined. Using ^{125}I heparin and chromatographic techniques, Dawes and Pepper (1982) speculated that each molecule of protamine binds one molecule of glycosaminoglycan irrespective of molecular weight. A heparin poly-L-lysine complex is formed by the nonstoichiometric binding of poly-L-lysine around a rigid heparin molecule. Jones et al. (1986) using fluorescence techniques have determined that the heparin protamine complex is probably a large cross linked stoichiometric structure. In vitro 1 mg protamine totally bound 1 mg of heparins as determined by HPLC, after the heparin-protamine complex was sedimented by centrifugation (Huang 1989).

B. In Vitro Interactions of Protamine and Heparin

Many in vitro experiments have been performed in order to elucidate the mechanism of the neutralization of heparin by protamine. Protamine sulfate binds to heparin and (1) either prevents the formation of the heparin AT III complex or (2) disrupts the activated

heparin-AT III complex (Hubbard and Jennings 1985). Neutralization of heparin in plasma by protamine represents an equilibrium reaction in which an excess of protamine is necessary to maintain heparin protamine complexes. If this excess is lost, through metabolism by a naturally occurring protaminase the complexes dissociate so that immediate antithrombin activity is reestablished (Shanberge et al. 1987). Okajima et al. (1981) have demonstrated that protamine dissociates heparin-AT III complexes by means of binding to heparin. In vitro, when protamine sulfate is added to heparinized human plasma, the activated heparin-antithrombin III complex is broken. AT III is freed and able to reestablish its original slow antithrombin activity. In neutralized plasma, antithrombin III exists in its native state and can be reactivated by any free heparin to become a potent inhibitor of factor Xa and factor IIa (Kitani et al. 1980).

Conventional heparin is neutralized more effectively in vitro by protamine than is low molecular weight heparin (Racanelli et al. 1985; Gram et al. 1988). Protamine chloride inhibited the clot based antifactor Xa activity 95% for conventional heparin and only 55% for low molecular weight heparin, while thrombin inhibition was completely neutralized. At equigravimetric concentrations of a low molecular weight heparin fragment and protamine, only 25% of the antifactor Xa activity was neutralizable by protamine sulfate. Protamine neutralization is not related to the anticoagulant potency of heparins. At concentrations of unfractionated heparin (0.17 μg), heparan sulfate (0.6 μg), or low molecular weight heparin (1.65 μg) which prolonged the APTT to a similar degree; low molecular weight heparin required more

protamine sulfate by weight than unfractionated heparin for complete neutralization of APTT activity. Neutralization of the anti Xa activity of unfractionated heparin required 4 μg of protamine sulfate to neutralize 1 μg of unfractionated heparin. 12 μg of protamine sulfate was required to completely neutralize the anti Xa activity of 1 μg of low molecular weight heparin (Hubbard and Jennings 1985).

Two hypotheses have been proposed to explain the different behavior of protamine with regard to heparin and low molecular weight heparin. This differential interaction may be could be related to the size of the anticoagulant molecule. Either protamine sulfate can bind to a long chain more easily or low molecular weight heparin induces the generation of protamine-resistant anti Xa activity in vivo (Thomas 1984).

Nonspecific interactions between protamine sulfate and plasma proteins such as PF4 and histidine rich glycoprotein inhibit the binding of protamine sulfate to heparins (Lane et al. 1987). Therefore, more protamine sulfate is required to neutralize heparin activity in a plasma system as compared to a purified system. Unfractionated heparin, heparan sulfate and low molecular weight heparins were all neutralized completely by 4 μg protamine sulfate for every 1 μg glycosaminoglycan when the antifactor Xa clotting assay was carried out using purified AT III in the absence of plasma.

Platelet factor 4, released from platelet granules, neutralizes the in vitro activity of heparin in a similar manner to protamine sulfate. 0.8 U/ml of heparin was totally neutralized by 15 μg/ml of platelet factor 4 and by 16 μg/ml of protamine sulfate in vitro. In

other studies, 1.0 U of heparin was neutralized by PF4 or protamine (15-20 μg/ml). This represents a 2:1 (protamine:heparin) ratio as measured by the kaolin cephalin clotting time, the thrombin clotting time, and modified Yin Wessler antifactor Xa clotting assays (Michalski et al. 1978). However, complexes formed between heparin and either platelet factor 4 or protamine are not equivalent. When heparinized plasma is treated with an excess of platelet factor 4, in contrast to protamine no large platelet factor 4-heparin-complexes form which are able to activate AT III (Shanberge et al. 1987).

The action of protamine and platelet factor 4 is complimentary. When a neutralizing dose of protamine was added to plasma supplemented with platelet factor 4 and heparin, the protamine displaced the platelet factor 4 from its complexes with heparin (Shanberge et al. 1987). This phenomenon has also been observed in rabbits by Cella et al. (1987) and may have clinical significance. Platelet factor 4 may stabilize heparin-protamine complexes even if excess of protamine no longer remains, since platelet factor 4 is not broken down in plasma as protamine is (Shanberge et al. 1987). The amount of protamine needed to neutralize heparin following extracorporeal bypass procedures may be less when large amounts of platelet factor 4 are released.

In vitro heparinized plasmas are readily neutralizable by protamine and PF4 in all assay systems. However neutralization of ex vivo heparinized plasmas are dependant upon the assay used to determine heparin activity. Ex vivo antifactor Xa activity differs from in vitro anti factor Xa activity. Only 1/2 of the antifactor Xa activity of injected heparin could be neutralized, even heparin (0.68 U/ml is

approximately 4.5 g/ml) obtained ex vivo was not completely neutralized by 320 μg/ml platelet factor 4. In addition only 50% of this heparin activity could be removed using a column of ECTEOLA-cellulose (Michalski et al. 1978). A possible explanation for the lack of neutralization of the anti Xa activity of injected heparin could be that heparin administered intravenously binds tightly to AT III while still retaining anti Xa activity or that intravenous injection of heparin releases cellular or intravascular components that interact with the anti Xa assay but not with other assays (Michalski et al. 1978). Hubbard and Jennings have speculated that heparan sulfate may be released upon heparin administration and that these glycosaminoglycans may be less neutralizeable by protamine sulfate (Hubbard and Jennings 1985).

C. In Vivo Interactions of Protamine and Heparin

Many animal models have been used to study the bleeding, antithrombotic effects and endogenous cellular interactions after neutralization of heparins by protamine. It has been demonstrated that protamine neutralizes the antiprotease, antithrombotic and antihemostatic effects of heparin to different degrees (Diness and Ostergaard 1986). In a rat tail bleeding model and vena caval ligation model, APTT and antifactor Xa, hemorrhagic and antithrombotic effects of heparin were completely neutralized at equigravimetric dosages of protamine sulfate (3 mg/kg i.v.). Equivalent doses with respect to antithrombotic potency of heparin and a low molecular weight heparin (LHN-1) was administered to rats. The hemorraghic effect of a large dose of LHN-1 in this experimental model was efficiently counteracted

by protamine sulfate, however higher doses of protamine sulfate were required to neutralize the antithrombotic effects. The hemorrhagic effects were neutralized even though some ex vivo activities were still present (Diness and Ostergaard 1986).

Similar results have been reported by Doutremepuich et al. (1985) and Racanelli et al. (1988) using low molecular weight heparin CY 216 in rabbits. Protamine did not neutralize all the biologic effects of CY 216, but completely neutralized the bleeding time when protamine was administered in the ratio of 1 antiheparin U to 2 IC anti Xa U of CY 216 (Doutremepuich et al. 1985).

Dogs have also been used to study whether total reversal of heparin immediately after endarterectomy has an adverse effect on the thrombogenicity of the endarectomized vessel wall (Chandler et al. 1982). Scanning electron microscopy revealed nearly total coverage of exposed collagen of the media with flattened platelets, scattered leukocytes, little fibrin and no thrombi. No differences were seen between those dogs which received protamine reversal and those which did not. Therefore total reversal of heparin does not increase the thrombogenicity of endartectomized vessels (Chandler et al. 1982).

It has been demonstrated in dogs that protamine neutralization of low molecular weight heparin causes less thrombocytopenia as compared to protamine neutralized heparin although neutropenia occurred equally in both groups. No differences were seen in eicosanoid or complement factors after protamine reversal of unfractionated heparin as compared to low molecular weight heparin (Lindblad et al. 1987). Protamine did not reverse the elevated antifactor Xa levels in low

molecular weight heparin anticoagulated dogs to the same degree as unfractionated heparin.

D. Toxicity of Heparin-Protamine Complexes

The cardiovascular changes which occur after protamine administration are dependant upon the route of administration and whether the heparin-protamine complex is formed before the protamine reaches the lungs. Plasma histamine levels were significantly higher after right atrial injection. Histamine is released as protamine traverses the lungs following right atrial injection and produces peripheral vasodilation. Histamine has been shown to have an H_2 receptor-mediated positive inotropic effect in man. It may be possible that protamine degranulates mast cells directly while protamine-heparin complexes do not. Heparin-protamine complexes may be involved in complement activation (Frater et al. 1982).

The administration of heparin followed by protamine results in a more severe cytopenia than protamine administration alone which lasts 30 to over 60 minutes. ^{125}I-labelled protamine in the presence of heparin binds tightly to platelets and granulocytes. Sequestration of the coated cells in the lungs results in transient granulocytopenia and thrombocytopenia (Al-Mondirhy, 1985).

A decrease in fibrinogen concentration has also been observed during cardiopulmonary bypass. Although the initial decline occurs during the first five minutes of cardiac bypass, another drop in fibrinogen concentration is always observed immediately after heparin neutralization (Gans et al. 1966). Cardiopulmonary bypass procedures often result in significant thrombocytopenia. Further decrease of the

platelets due to protamine could pose a serious threat in the immediate postoperative period (Al-Mondirhy 1985).

Dose dependant activation of the complement system by heparin-protamine complexes has been shown in man. A significant increase in activated components of the complement system (C3d, C3a, C4a and C4d) were obtained in patients after the administration of protamine sulfate (Best et al. 1984; Kirklin et al. 1986). Peak levels of C3a and C4a were seen in samples taken 10 minutes after protamine administration. C4d peak levels were obtained at 5 hours. The level of C3a, 3 hours after cardiopulmonary bypass with protamine administration has been shown to be a risk factor for postoperative morbidity (Kirklin et al. 1986). Protamine acts as a substrate for C reactive protein, a potent activator of the complement system at C1 (Frater et al. 1984). Also activation of the classic complement pathway occurs by protamine-heparin polycation-polyanion complexes. In vitro combination of protamine sulfate and heparin, but neither heparin nor protamine alone resulted in a increase in C_{3a} and C_{4a} levels (Kirklin et al. 1986). Activation of complement may cause an increase in vascular permeability and could contribute to the pathogenesis of noncardiogenic pulmonary edema (Best et al. 1984).

Adverse reactions which occur after the administration of protamine include:hypotension, anaphylactoid responses and catastrophic pulmonary vasoconstriction. Rapid administration of protamine results in pulmonary vasoconstriction. Rapid administration of protamine results in systemic hypotension in most patients (Horrow 1985), however Coulon et al. (1982) speculate that even a small dose of protamine

sulfate will change systolic blood pressure. Anaphylactoid responses to protamine are characterized by edema of the skin, mucosa, viscera decreased systemic vascular resistance and bronchospasm (Horrow 1985). Antihistamines, aminophylline and steroids are employed in the treatment of anaphylactic reactions after protamine administration (Stoelting 1983). Catastrophic pulmonary vasoconstriction (type III) is accompanied by right ventricular dilation, pulmonary arterial hypertension, decreased left ventricular filling pressure and systemic hypertension, decreased left ventricular filling pressure and systemic hypotension. This syndrome has occurred after minimal doses of protamine (10 mg) and may resolve spontaneously (Horrow 1985). Treatment with isoproterenol or epinephrine has been successful in the reversal of these effects (Lowenstein et al. 1983).

IV. CLINICAL IMPLICATIONS OF PROTAMINE NEUTRALIZATION OF HEPARINS

A. Protamine Neutralization of Heparin in Patients

Reversal of the anticoagulant activity of heparin has contributed significantly to the field of cardiovascular surgery. Monitoring of a patient's state of anticoagulation during this procedure is crucial (Bull et al. 1975) since the amount of heparin required to produce an arbitrary prolongation in clotting times has been shown to vary 3 fold from patient to patient and the rapidity with which the administered heparin dose disappears from the blood may vary fourfold. 76% of patients receiving common doses of heparin during cardiopulmonary bypass were found to have plasma heparin levels that were potentially too low (<1.7 U/ml; recommended level is 2 U/ml), (Umlas et al. 1983).

During cardiopulmonary bypass, high doses of heparin (3 mg/kg i.v.) are used to prevent thrombosis in patients amd to prevent clot formation within the heart lung machine (Shanberge et al. 1986). Since such high doses of heparin are used which result in hemorrhage, neutralization by protamine sulfate is necessary.

Many different protocols have been used for the protamine neutralizaton of heparin activity by protamine after cardiopulmonary bypass. Some of these protocols are based on arbitrary amounts of protamine to neutralize a given amount of heparin while others are calculated from known heparin-protamine interactions. The inability to measure heparin levels accurately and rapidly has prevented the accurate prediction of the minimum dosages necessary to reverse heparin (Umlas et al. 1983).

Reversal of heparin in patients has been shown to follow the same in vitro pattern of dissociation of the neutralization of the anti Xa and anti IIa activities were observed in vitro (Racanelli et al. 1985) Incomplete neutralization of the lipoprotein lipase, hepatic triglyceride lipase and factor Xa activities of a low molecular weight heparin by protamine chloride has been shown in patients (Harenberg et al. 1985). Similarly, after the administration of protamine chloride to patients who received either (7,500 or 18,750) anti Xa units of CY 216, the inhibition of factor Xa was reduced to approximately 40 % inhibition. Similar results were obtained with a different low molecular weight heparin of similar molecular weight range. Therefore the molecular weight range of a low molecular weight heparin seems to be a more important determinant for protamine neutralization than the

method of preparation of the heparin (Harenberg et al. 1986). In vitro, anti Xa and anti IIa activities and APTT activity of unfractionated heparin were neutralized with a gravimetric protamine:heparin ratio of 1.6 to 1, 1.3 to 1 and 2 to 1 respectively. Anti IIa and APTT activity induced by a low molecular weight heparin PK 10169 returned to baseline completely at a protamine:heparin ratio of 1 and 2 respectively while anti Xa activity was not completely neutralized at a gravimetric protamine: heparin ratio of 5 to 1.

Since the direct cause of blood loss is not always easy to determine, abnormal bleeding during or after cardiopulmonary bypass has been a serious problem. Residual heparin (due to an inadequate neutralizing dose of protamine), anticoagulation induced by excessive protamine, thrombocytopenia and a variety of other conditions such as disseminated intravascular coagulation, platelet dysfunction and fibrinolysis have all been postulated as causes for abnormal bleeding in patients undergoing cardiopulmonary bypass (Umlas et al. 1983).

Bleeding times have been reported to be prolonged in patients after administration of unfractionated heparin and CY 216. Heparins may interact with platelets at the surface of the vessel wall by some unknown mechanism and thereby cause bleeding (Harenberg et al. 1986). A platelet effect is suspected to prolong the bleeding time since substances which influence platelet function prolong this test whereas vitamin K antagonists such as coumadin have no effect of bleeding time (Harenberg et al. 1986).

In a study by Massonet-Castel (1986) patients which were treated prophylactically with heparin, exhibited normal or only slightly

increased bleeding. In 3 patients given protamine because of hemorrhage, mean anti Xa and anti IIa levels were 2.3 an 0.54 U/ml respectively before and 1.32-0.06 U/ml respectively after neutralization. A correlation between bleeding, anti Xa and anti IIa activities was not clearly evident. The dose of protamine which was used to neutralize low molecular weight heparin PK 10169 was 2 mg for every 1 mg of PK 10169 as compared to 1.3 mg of protamine for every 1 mg of unfractionated heparin. Whether the difficulty in reversing the anti Xa activity constitutes a clinical drawback for the use of low molecular weight heparins remains to be seen (Massonet-Castel et al. 1986).

B. Heparin Rebound

In 1956, Kolff et al. first described heparin rebound as a "treacherous phenomenon in which heparin is neutralized by protamine sulfate and the clotting time becomes normal in a matter of minutes. However protamine seems to be eliminated from the blood before heparin is, thus leaving the heparin uncovered as demonstrated by protamine" (Ellison et al. 1974). Heparin rebound is defined as the reappearance of hypocoagulability after adequate neutralization (Pifarre et al. 1981) which is restored to normal by further protamine administration (Gollub et al. 1967). Protamine neutralization of the heparin activation of antithrombin III in plasma, in vitro, represents an equilibrium reaction in which by mass action, heparin remains complexed only in the presence of an excess of protamine (Kitani et al. 1980).

Many possible explanations have been suggested for the rebound of anticoagulant activity after protamine administration which include: heparin may be released by red blood cell breakdown, heparin may escape

from the circulation into the extravascular space and return via the lymphatics and thoracic duct to the circulation many hours after protamine has been administered and cleared. Heparin rebound may be more commonly observed with hypothermia, since hypothermia during cardiopulmonary procedures procedure produces a decrease in the elimination of heparin which may contribute to the phenomenon of heparin rebound (Cohen et al. 1977).

The whole blood activated clotting time is widely used to monitor heparin anticoagulation during cardiopulmonary bypass and to predict the neutralizing dose of protamine sulfate (Piffare et al. 1981). Recent reports suggest that this assay system may not give a true reflection of the plasma heparin levels and may give inaccurate predictions of the required protamine dose (Esposito et al. 1983). The accurate diagnosis of heparin induced bleeding is difficult to determine and it is not uncommon to empirically administer additional doses of protamine in the face of post bypass bleeding, especially if the APTT is prolonged (Ellison et al. 1974).

Postoperative heparin rebound was investigated in 50 patients undergoing cardiopulmonary bypass who received 2 mg/kg of heparin preoperatively. The overall mean ratio of protamine to heparin administered was 1.25:1. One hour after heparin was completely neutralized, 26 patients (52%) had circulating heparin and required an additional dose of protamine averaging 70 mg (Piffarre et al. 1981). In another clinical situation, heparin rebound was observed in 29% of cases which correlated with circulating load (i.u. x 10^3) of heparin administered. The ratio of protamine sulfate to circulating load of

heparin was lower than 1.6 in all cases which exhibited rebound (Kesteven et al. 1986). In another study, 20 out of 40 patients exhibited heparin rebound (Gollub 1967).

The occurrence of heparin rebound depends upon the ratio of protamine to heparin. Low incidence of heparin rebound was reported in patients who received an excess of protamine after cardiopulmonary bypass as compared to those who received the minimum amount as determined by titration methods (Shanberge et al. 1986). In another study (Ellison et al. 1974), every patient who received the minimal dose of protamine exhibited, rebound phenomenon at some time between one and five hours after neutralization. In three cases rebound was associated with clinical bleeding which necessitated additional protamine. In contrast none of the patients who received a larger dose of protamine exhibited rebound (Gollub 1967). He suggested that patients be given the dose of protamine that is indicated by titration plus an additional 10 mg of protamine per liter of blood volume (usually 50 mg of additional protamine). Using this regimen, there was no danger of excess protamine administration and there was a noticeable decrease in the incidence and magnitude of heparin rebound in this study.

Other factors which may cause heparin rebound include increased heparin binding to plasma proteins, the specific anion bound to protamine and the length of the bypass procedure. Heparin bound to other proteins such as alpha$_2$ macroglobulin may be able to activate any fresh antithrombin III produced by the patient or administered through infusion of blood or plasma (Shanberge and Sridhar 1981). Protamine sulfate inactivates those heparin fractions that are bound to AT III

but not those bound to alpha$_2$ macroglobulin. The stability of protamine derivatives is not influenced by the specific anion which is bound to them. But rather, differences are acquired in the course of the manufacturing procedure involved in their preparation which influence the stability of the protamine preparation. Commercial protamine chloride is stable in the presence of plasma enzymes, whereas the sulfate is degraded (Benayahu and Aronson 1983). Heparin rebound could be reproduced with the sulfate but not with the chloride anion. The length of cardiopulmonary bypass influences the amount of protamine required postoperatively (Pifarre et al. 1981).

In vitro heparin is neutralized at a gravimetric ratio of 1:1; therefore in vitro studies may seriously underestimate the required dose of protamine sulfate following surgery (Shanberge et al. 1986). In clinical situations 1.3 mg of protamine is used to neutralize 100 U of heparin (Massonnet-Castel et al. 1986). Other investigators have questioned the necessity of protamine reversal of heparin following bypass surgery, (Castenda et al. 1967). It may be advantageous to prolong the period of heparinization of the patient into the early postoperative period, if bleeding is not profuse.

C. Clinical Trials With Low Molecular Weight Heparins

Over the last decade, there has been a considerable amount of interest in the clinical use of low molecular weight heparin (Messmore et al. 1985). Low molecular weight heparins have been studied in randomized clinical trials to evaluate their effectiveness as well as the risk/benefit ratio of their routine use in postoperative prevention of deep venous thrombosis and pulmonary embolism (Samama et al. 1989).

Several clinical studies have been conducted in which a low molecular weight heparin has been compared with standard unfractionated heparin following general and orthopedic surgery and in general medicine. Studies have also been performed in which low molecular weight heparins were compared to a placebo. A low molecular weight heparin, Fragmin^{R} was studied in patients undergoing general surgery by Bergvist et al. (1986). Low rates of thrombosis were found in both treatment groups, 6% versus 4 % and no statistical significance between the drugs was observed. In a trial by Kakaar (1985), using the low molecular weight heparin, Fraxiparine^{R}, there was a statistically significant reduction in postoperative venous thrombosis in patients who received Fraxiparine^{R}. Similarly, in a study by Encke and Breddin (1988) where control groups received heparin 5000 U twice or three times daily, a significant difference was observed which supported the greater efficacy of Fraxiparine^{R}. In other studies by Koller et al. (1986) and Schmitz-Huebner et al. (1984) thrombosis rates were low in both groups however no statistical significance between groups was observed.

Studies have shown that fixed-dose subcutaneous heparin administration is partially effective in the prevention of deep vein thrombosis after total hip replacement surgery (Bergvist et al. 1980) and adjusted low-dose heparin administration leads to significant improvement (Leyvraz et al. 1983). It was therefore important to determine if low molecular weight heparins would be more useful during orthopedic surgery than heparin. In studies of patients undergoing hip surgery, the incidence of thrombosis was substantially reduced by low

molecular weight heparin, Enoxaparin^{R}, from 42 % to 12 % as compared to placebo (Turpie et al. 1986). These results may be compared with those obtained after a single injection of 40 mg of Enoxaparin^{R} to patients undergoing hip surgery in a double blind study. The frequency of thrombosis as assessed by phlebography was significantly lower with low molecular weight heparin than with three injections of 5000 IU of heparin (Planes et al. 1988).

Prophylactic heparin is often used in hospitalized medical patients at high risk for thromboembolism. Two hundred seventy patients confined to bed rest and over 65 years of age were studied in a randomized double-blind trial (Dahan et al. 1986) comparing 60 mg of Enoxaparin^{R} to a placebo over a 10-day period. Enoxaparin^{R} produced a statistically significant ($p<.05$) reduction in isotopically detectable venous thrombosis (3% versus 9%). Twelve patients died during the trial (six in each group) and autopsy revealed three fatal recent pulmonary embolism in the placebo group and one in the treated group (Samama et al. 1989).

The incidence of bleeding varied markedly in the clinical trials of low molecular weight heparins. This variation may be due to differences in the criteria used to define clinical important bleeding and also was dependant on the doses selected for the studies. In three out of five studies, the incidence of bleeding was significantly greater in the low molecular weight heparin group than in the unfractionated heparin group (Bergvist et al. 1986; Koller et al. 1986; Schmitz-Huebner et al. 1984). In the study reported by Koller, in 1986, there was a 48 % rate of bleeding in 23 patients who received

Fragmin^{R} compared to a 10 % rate in patients who received unfractionated heparin. This trial was terminated because of excess hemorrhage in the experimental group. The incidence of bleeding was low for clinical trial of ORG 10172 heparinoid and for Fraxiparine^{R}. There was a 2 % rate of bleeding as compared to a 4 % rate for placebo. Kakaar (1985) reported a 5 % rate of bleeding in patients undergoing abdominal surgery who received Fraxiparine^{R} compared to a bleeding rate of 3.5 % in patients who received unfractionated heparin. A correlation (r=.54) between ex vivo anti Xa levels produced by low molecular weight heparin and clinically important bleeding was reported.

Low molecular weight heparin Enoxaparin^{R} has been studied in 15 patients who underwent surgery with extracorporeal circulation which required reversal by protamine sulfate (Massonnet-Castel et al. 1986). In vitro, anti Xa and anti IIa activities and APTT activities of heparin were neutralized by a protamine:heparin ratio of 1.6, 1.33 and 2 respectively. Anti IIa activity and APTT induced by Enoxaparin^{R} were completely neutralized a protamine to heparin ratio of 1 and 2 respectively, while anti Xa activity was incompletely neutralized at a ratio of 5. In patients which did not receive protamine a good correlation was observed between doses of Enoxaparin^{R} infused, anti IIa plasma levels and blood loss. In patients who received protamine because of hemorrhage, bleeding was stopped, however a poor correlation between bleeding, anti Xa or anti IIa activities was observed (Massonnet-Castel 1986).

CHAPTER II

STATEMENT OF PURPOSE

Protamine neutralization of the hemorrhagic effects of heparin has contributed to the widespread therapeutic acceptance of heparin. The routine use of heparin during surgical procedures such as cardiopulmonary bypass could not have been possible without the use of protamine as an antagonist. Although protamine has been used for decades to antagonize the bleeding effects of heparin, the biochemical and pharmacologic aspects of this interaction are still not completely understood. In addition, heparin protamine complexes are known to exert a variety of toxic effects which are not fully investigated at this time. Furthermore, with the development and increased clinical use of low molecular weight heparins, it is important to better characterize the interaction of protamine with heparins.

Although protamine is administered primarily to neutralize the bleeding effects of heparin, this antagonist also influences several other activities of heparin. In addition, protamine has been shown to prolong clotting tests (Gans 1962), produce thrombocytopenia (Al-Mondhiry 1985), accelerate fibrin monomer polymerization and inhibit thrombin (Cobel-Geard 1983). Since administration of protamine alone exerts effects on the hemostatic system, the study of the neutralization of heparin activity by protamine becomes complicated. It is the purpose of this dissertation to compare the protamine neutralization of heparin and a well characterized

low molecular weight heparin (CY 216), in terms of their anticoagulant, antiprotease, antithrombotic and pharmacodynamic actions.

These studies provide integrated data on the differences between the neutralization profiles of heparin and a low molecular weight heparin (CY 216). Such information is needed to optimize the use of protamine especially for the neutralization of low molecular weight heparins. A better understanding of protamine-heparin interactions as investigated in this study may lead to safer use of these agents.

A series of in vitro assays used to study the protamine neutralization of the heparins included global clotting assays and specific chromogenic substrate based assays. Use of these assays allowed for an integrated approach to profile the potency, sites of action and degree of neutralization of these heparins by protamine.

Thrombosis involves a disruption of the delicate balance between blood vessels, platelets and coagulation factors. To address this area, two defined models of thrombosis, a modified version of the classical Wessler stasis thrombosis model (Wessler 1974) and a laser-induced model of thrombosis as developed by Breddin (1982) were used. Pharmacologically defined studies were carried out to approximate the current clinical use of heparins and focus on the antagonism of the antithrombotic effects of heparins by protamine.

Since hemorrhage also is a multifactorial process involving many hemostatic components, two animal models of blood loss which have been widely utilized in the study of heparins and other anticoagulant drugs, were used to study the antagonism of heparin and CY 216 by protamine. The relevance of ex vivo anticoagulant and antiprotease activities to the

observed blood loss and its antagonism was extensively addressed in this investigation.

The pharmacodynamic time course parameters of both heparin and CY 216 prior to and after the administration of protamine were determined utilizing an established pharmacokinetic model. A unique feature of this study is the use of a nonhuman primate (maccaca mulatta) model whose hemostatic profile has been found to approximate the human hemostatic system (King 1989). Intravenous and subcutaneous pharmacodynamic studies provided timely information on whether protamine alters the time course parameters of heparin or CY 216 and whether the observed protamine neutralization of heparin and CY 216 was assay dependent. This information is of crucial importance in the understanding of heparin rebound. This study also represents the first comprehensive approach to establish the differences in the in vitro protamine neutralization profiles and pharmacologic behavior of heparin and a low molecular weight heparin.

The biochemical and pharmacologic experiments included in this investigation have been used as model systems to investigate the interactions of heparin with protamine. This dissertation thus emphasizes the need of an integrated approach to obtain crucial pharmacologic data on the interactions of polycomponent drugs. This may have a direct impact on the clinical management of patients administered heparin and protamine.

CHAPTER III

MATERIALS AND METHODS

I. MATERIALS

A. Antithrombotic Drugs

1. Heparin

Porcine mucosal heparin (lot H503) was obtained from Choay Institute (Paris, France). The mean molecular weight of heparin as determined by a computerized gel permeation chromatographic system was 11,000 daltons. The anticoagulant potency of this heparin is 165 IU/mg. The analytical and molecular weight profiles of heparin (lot H 503) are shown in Appendices II and III respectively.

2. Low Molecular Weight Heparin

CY 216 (lot P 533 XH), a low molecular weight heparin, produced from porcine mucosal heparin by nitrous acid depolymerization was obtained from Choay Institute (Paris, France). This low molecular weight heparin has a mean molecular weight of 5,000 daltons and is characterized by a narrower molecular weight distribution than heparin. Eighty per cent of the chains of CY 216 have a molecular weight within the range of 2,400-7,200 daltons which corresponds to 4 to 12 disaccharide units. Dodeca-, tetradeca-,and hexadecasaccharidic chains account for 50% of the molecular species composing CY 216. In addition 25% of the chains contain a specific pentasaccharide sequence that enables them to bind to AT III with high affinity (Lormeau 1989). The potency of CY 216 is 226 IC AXa U/mg and 58 USP U/mg. The analytical

and molelcular weight profiles of CY 216 (lot P 533 XH) are shown in Appendices IV and V respectively.

B. Protamine Sulfate

Protamine sulfate obtained from Choay Institute was produced from salmon sperm and contains 138 UAH/mg. The mean molecular weight of this protamine as determined by HPLC is 4,000 daltons. The analytical and molecular weight profiles of protamine sulfate are shown in Appendix VI and VII respectively.

C. Spermidine

Spermidine (N-(3-aminopropyl)-1,4-butanediamine) was purchased from Sigma Chemical Co. (St. Louis MO).

D. Reagents

All reagents obtained for use in the assays were of analytical grade. All drugs and reagents used in the studies were stored in desiccated storage containers at appropriate temperatures.

1. Clotting Assays

Automated APTT, a rabbit brain phospholipid reagent containing micronized silica as an activator, was obtained from General Diagnostics (Morris Plains, NJ) in lyophilized form and reconstituted with distilled water according to manufacturer's instructions.

Fibrindex^{R} human thrombin was purchased from Ortho Diagnostic Systems, Inc. (Raritan NJ) in lyophilized form and was reconstituted with saline to obtain a 5 NIH U/ml solution. The thrombin solutions were adjusted using normal human plasma to give a 20.0 $\pm$ 2.0 second clotting time using the thrombin time assay. Aliquots of thrombin were frozen at -70^{o}C for periods of not more than 6 weeks.

Heptest^{R} reagents were obtained from Haemachem (St. Louis, MO) and included bovine factor Xa and Recalmix^{R}. This reagent contains optimal concentrations of calcium chloride and brain cephalin in a bovine plasma fraction. The reagents were obtained in lyophilized form and reconstituted according to manufacturer's instructions.

2. Amidolytic Assays

Reagents used for amidolytic anti Xa and amidolytic anti IIa assays include:

Buffer consisting of 0.050 M Tris (hydroxymethyl aminomethane, Trizma^{R} HCL,;Sigma Chemical Co., St. Louis, MO) 0.175 M sodium chloride (Sigma Chemical Co.), and 0.0075 M EDTA (Sigma Chemical Co.) were prepared at pH 8.4 at room temperature.

Bovine Factor Xa was obtained from Enzyme Research Laboratories (South Bend, IN) in lyophilized form. This preparation was reconstituted in 1.0 ml Tris buffer, diluted 1:4 in saline and stored at-70°C for periods up to 6 weeks. Factor Xa was calibrated for the anti Xa amidolytic assay by adjusting the above solution to produce a change of 0.600 to 0.700 mau (maximal activity unit)/minute in the assay blank at 405 nm.

Stock solutions (10 NIH U/ml) of Fibrindex^{R} human thrombin were reconstituted with saline and stored at -70°C. A working solution of thrombin was calibrated for the anti IIa amidolytic assay by adjusting a 10.0 NIH U/ml solution to produce a change of 0.600 to 0.700 mau (maximal activity unit)/minute in the assay blank at 405 nm.

Chromogenic substrates used in the amidolytic assays were purchased from American Diagnostica (Greenwich, CT). Spectrozyme Xa^{R}

(CH_3OCDHG-Gly-Arg-pNA) was reconstituted with distilled water to obtain a final concentration of 2.5 mM and was used in the amidolytic anti Xa assay. Spectrozyme TH^R (HDCT-Ala-Arg-pNA) was reconstituted with distilled water to obtain a final concentration of 1.0 mM and was used in the amidolytic anti IIa assay.

3. Pooled Plasma Preparations

For the in vitro studies, human plasma was obtained from the Loyola University Medical Center Blood Bank. All plasma preparations were screened for the presence of HIV (Abbott Laboratories, Chicago, IL) and hepatitis B virus (Abbott Laboratories, Chicago, IL) and found to be negative. The fresh frozen plasma packs obtained from 5 donors were thawed at 37° C, pooled (lot numbers for pool I: 1190423, 1190468, 1190464, 1190322, 1190469, lot numbers for pool II: 1317061, 1192592, 3142912, 1247780, 1196245) aliquoted in 10 ml volumes and frozen at-70°C for a period of not more than 2 months. Immediately before its use, pooled plasma was thawed at 37° C.

a. Normal Monkey Plasma Preparation (NMP)

Primates were anesthetized with ketamine HCL (10 mg/kg i.v.) and blood was drawn from the popliteal vein using a 21 gauge 3/4 inch butterfly needle and centrifuged at 4°C, 1200 x g for 20 minutes. Plasma from a minimum of 5 primates was pooled and aliquoted in 10 ml volumes. The pooled monkey plasma (NMP) was frozen in plastic tubes at -70°C for not more than 2 months.

b. Normal Rabbit Plasma Preparation (NRP)

White New Zealand rabbits were anesthetized with intramuscular injections of xylazine (20 mg/kg) and ketamine (80 mg/kg). After

induction of anaesthesia, blood was drawn by cardiac puncture using a Vacutainer^{R} technique into tubes containing 3.8 % citrate (1:10). The blood was centrifuged at 4°C, 1200 x g for 20 minutes. Plasma from a minimum of 5 rabbits was pooled and aliquoted in 5 ml volumes. The pooled rabbit plasma (NRP) was frozen in plastic tubes at -70°C for not more than 2 months.

c. Normal Rat Plasma Preparation (NratP)

Rats were anesthetized with ketamine HCL (40 mg/kg i.m.) and Xylazine (20 mg/kg i.m.). Blood was drawn from the inferior vena cava using a 22 gauge needle and a syringe into tubes containg 3.8 % citrate (1:10). 5 ml of blood was drawn from each rat. The blood was centrifuged at 4° C, 1200 x g for 20 minutes. Plasma from a minimum of 5 rats was pooled and aliquoted in 5 ml volumes. The pooled rat plasma (NratP) was frozen in plastic tubes at -70°C for not more than 2 months.

4. Thrombogenic Agents

Thrombogenic challenges used in the modified rabbit stasis thrombosis model included: prothrombin complex concentrate (PCC) and Russell's viper venom (RVV). Konyne^{R} brand of human prothrombin complex concentrate containing factors II, VII, IX and X was obtained from Cutter Laboratories, Lot 3125-50-3. PCC was reconstituted with saline to 50.0 U/ml. Russell's viper venom (RVV), lot 027F9461 obtained from Sigma Chemical Co. and was reconstituted to 0.1 U/ml with saline immediately before use.

5. Anaesthetics

Vetalar^{R} (ketamine hydrochloride) anaesthetic was obtained from Parke-Davis, (Morris Plains, NJ) as a 100 mg/ml solution. After intramuscular injection, Vetalar^{R} produces rapid analgesia with mild cardiac stimulation and respiratory depression. Rompun^{R} (xylazine) analgesic was obtained from Bayvet Division of Miles Laboratories, Inc. (Shawnee, KS) as a 100 mg/ml solution. Xylazine is a non-narcotic sedative, analgesic and muscle relaxant.

Beuthanasia-D^{R} euthanasia solution was obtained from Schering Corporation (Kenilworth, NJ). Each ml of beuthanasia contains 390 mg pentobarbital sodium and 50 mg phenytoin sodium in addition to inactive ingredients.

E. Animals

A colony of mature rhesus monkeys (macaca mulatta) consisting of 2 males and 10 females, housed in the animal research facility of Loyola University Medical Center, were used for the pharmacodynamic time course studies. The animal research facility is accredited by the American Association for the Accreditation of Laboratory Animal Care and the health of the primates was monitored by a licensed doctor of veterinary medicine. All primates were maintained on a standard diet of Purina monkey chow, fruit, molasses sandwiches, had free access to water and were exposed to a regular 12 hour light/dark cycle. The primates weights ranged from 5.7 to 14.2 kg. The average weight of the primates was 8.6 $\pm$ 2.2 kg (mean $\pm$ S.D.). No differences between the coagulation parameters of male and female primates are observed (Schalm 1975) and females in menses were not included in the studies.

Male New Zealand rabbits (oryctolagus cuniculus) ranging in weight from 2.5 to 3.0 kg were obtained from Langshaw Farms (Augusta, MI). Rabbits were housed in the Loyola University Medical Center animal research facility. Environmental conditions included a 12 hour light/12 hour dark cycle. They were fed a standard diet of Wayne^{R} Rabbit Ration and water ad libitum. All animals were allowed to acclimate to the facilities for a minimum of 7 days post transport.

Male Sprague Dawly rats (rattus norvegicus) ranging in weight from 240 to 260 g were obtained from Harlan (Indianapolis, IN). The rats were housed in the Loyola University Medical Center animal research facility. Environmental conditions included a 12 hour light/12 hour dark cycle. They were fed a standard diet of Wayne rodent lab blocks. All animals were allowed to acclimate to the facilities for a minimum of 7 days post transport.

Male Wistar rats (rattus norvegicus) ranging in weight from 200 to 250 g, used for the laser-induced thrombosis model, were housed in the accredited animal research facility of J.W. Goethe University, Frankfurt, West Germany. All animals were allowed to acclimate to the facilities for a minimum of 7 days post transport.

F. Analytical Instrumentation

Waters^{TM} 840 Data and Chromatography Control Station manufactured by Millipore Corporation (Millford MA) was used to run high performance liquid chromatography (HPLC) analyses. This system uses Waters Expert ^{TM} chromatography software (Version 6.2) and consisted of a Waters^{TM} WISP, a Waters^{TM} 510 HPLC pump, a Waters^{TM} 410 Differential Refractometer and a Waters^{TM} 490 Programmable Multiwavelength Detector.

TSK 2000 SW VIII column Beckman Instruments Inc. (Arlington Heights IL).

Fibrometer^{R}, manufactured by Becton Dickinson and Co. (Rutherford, NJ) is an electromechanical instrument used for clot detection at 37°C.

Spectrophotometer Model 35^{R} and Model DU7^{R} manufactured by Beckman Instruments, Inc.

Hycel Cell Counter 300, (Houston TX)

Argon Laser CR2^{R} manufactured by Coherent (Palo Alto, CA).

Microscope with interference contrast objectives and condensers and a 100 x water immersion objective was supplied by Leitz Wetzlar, Germany.

Surgical instruments used include scalpels with No. 20 blades, Metzenbaum dissecting scissors, tissue forceps and haemostats.

Accutemp^{R} Surgical Cautery manufactured by Concept, Inc. (Clearwater, FL) was used to cut through tissue while producing a minimal amount of bleeding.

An IBM PC-XT computer and a Blue Chip IBM compatible PC were used for word processing, graphics and statistics. The software used included: Word Perfect 4.2^{R} (Word Perfect Corporation, Orem, Utah), Sigmaplot^{R} (Jandel Scientific, Sausolito, CA), PCNONLIN^{R} (Statistical Consultants, Lexington, KY), and Systat^{R} (Systat Inc., Evanston IL).

II. METHODS

A. Physiochemical Profiling of Heparin, CY 216 and Protamine

1. Molecular Weight Determination

In order to determine the molecular weight profile of heparin, CY 216 and protamine, gel permeation chromatography (Losito 1981) was performed on a Waters 840 Data and Chromatography and Control Station which utilized Waters Expert™ chromatography software. The system calculated not only mean molecular weight, but also values which indicate molecular weight distribution. These values were calculated from the chromatographic characteristics of standards with similar molecular composition on a TSK 2000 SW VIII column. Thus prior to use, the column was calibrated with anionic polymers consisting of sulfated glucuronic acid, uronic acid and glucosamine (Emanuele 1988). Values for retention time (RT) and polymer dispersity (D) were determined, and used along with molecular weights of the standards to calculate a calibration curve. This curve was calculated by third polynomial regression and yielded polynomial coefficients (D_o, D_1, D_2, D_3) that characterized the average standard curve. The following equation was used:

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

The molecular weight of the heparin and CY 216 were determined from their chromatographic characteristics under the following conditions:

Columns: TSK 2000 SW VIII (Beckman)

Mobile Phase: Na_2SO_4 0.5 M

Detector: UV 205 nm

Flow Rate: 1 ml/min

Sample: 20 μl of heparin or CY 216 (10 mg/ml)

The molecular weight of protamine was determined under the following conditions:

Columns: TSK 2000 (Beckman)

Mobile Phase: Na_2SO_4 0.5 M

Detector: UV 205 nm

Flow Rate: 1.0 ml/min

Sample: 20 μl of protamine (10 mg/ml)

2. NMR spectra

The ^1H NMR spectra of heparin, CY 216, protamine and a heparin-protamine complex were obtained in collaboration with Professor Casu of Institute Ranzoni, Milan, Italy. A Bruker^R AC 200 nuclear magnetic spectrometer was used to obtain the ^1H spectra. Each agent was dissolved in deuterated water (D_2O) at 20% and a full span spectra was recorded according to a method described previously (Casu 1984).

To prepare a complex between heparin and protamine sulfate, 20 mg of protamine was suspended in 0.5 ml of 0.1 N DCl, mixed well and 1 mg of heparin was added. This solution was incubated for 1 hour and a ^1H NMR spectrum of this mixture was obtained. A control spectrum of protamine was also determined using the same conditions.

B. <u>In Vitro Methods</u>

1. Clotting Assays

The activated partial thromboplastin time (APTT), thrombin time and Heptest^R are global clot based assays. These tests are sensitive to many factors which can influence the coagulation system by inhibiting a variety of enzymatic processes. The end point of all clotting

tests is the formation of fibrin which is detected by a fibrometer, an instrument which mechanically detects clot formation.

APTT is a test used to screen the intrinsic pathway of coagulation (factors XII, XI, IX, VIII, X, V, II, I) and for monitoring heparin therapy. It is based on the measurement of time to clot after citrated platelet poor plasma has been activated by a platelet substitute (phospholipid), silica activator and calcium chloride. The assay was performed by mixing 100 μl of citrated plasma with 100 μl of automated APTT reagent^{R} and incubating for 5 minutes at 37^{o}C. 100 μl of calcium chloride (0.025 M) prewarmed to 37^{o}C was then added. The time to clot was determined immediately by a fibrometer. Any clotting time which was prolonged more than 150 seconds was reported as >150 seconds which is the upper limit of the linear range of this assay.

Thrombin clotting time measures the conversion of fibrinogen to fibrin based on the time to clot after the addition of a known amount of thrombin to plasma. The assay was performed by incubating citrated plasma (200 μl) at 37^{o}C for 3 minutes at which time 100 μl of 5 U/ml human thrombin was added. The time to clot was determined immediately by a fibrometer. Any clotting time which was prolonged more than 150 seconds was reported as >150 seconds which is the upper limit of the linear range of this assay.

Heptest^{R} was performed according to manufacturer's instructions. The fundamental principle of the Heptest^{R} heparin assay involves the ability of heparin or its derivatives to catalyze the inactivation of exogenous bovine activated factor X by antithrombin III in the presence of naturally occurring antagonists in the plasma sample. Heptest^{R}

measures the anticoagulant property of heparin through the combined anti Xa/anti IIa actions of the drug. Citrated plasma (100 μl) was incubated with bovine factor Xa (100 μl) for exactly 120 seconds, at which time 100 μl of Recalmix^{R} was added to the mixture and immediately the time to clot was determined by a fibrometer. Any clotting time which was prolonged more than 300 seconds was reported as >300 seconds which is the upper limit of the linear range of the Heptest^{R}.

2. Amidolytic Assays

Amidolytic assays are characterized by an enzymatic reaction in which an oligopeptide synthetic substrate of semi-specific structure is cleaved by a given enzyme to release an attached chromophore (para nitroaniline). Enzyme activity is determined by measuring the increase in absorbance of the free chromophore which is generated, in comparison to the original substrate, per minute at 405 nm (Fareed 1980, 1983; Hoppensteadt 1985) One advantage of amidolytic assays is the ability to directly quantitate specific enzymes rather than indirectly as in the case of the global assays. This technique, however, measures amidolytic activity on a semi-specific substrate which may not directly correspond to the specific function of the enzyme in coagulation processes.

The amidolytic anti Xa assay provided a measure of absolute heparin concentration which is proportional to the inhibition of factor Xa by heparin. The assay was performed in the following manner: 375 μl of buffer (pH 8.4) was equilibrated with 25 μl of plasma sample at 37^{o}C. 50 μl of 0.5 nkat/ml bovine factor Xa was added and allowed to react for exactly 2 minutes, at which time 50 μl of substrate (Spectro-

zyme Xa 2.5 mM) was added. The change in absorbance was recorded at 405 nm for one minute. % Inhibition of factor Xa activity was calculated as shown below.

$$\% \text{ Inhibition} = \frac{\text{plasma sample delta A } 405 \text{ nm/min}}{\text{plasma blank delta A } 405 \text{ nm/min}} - 1 \times 100$$

The amidolytic anti IIa measures the inhibition of thrombin (factor IIa) which is proportional to the heparin concentration. The assay was performed in the following manner: 400 μl of buffer pH 8.4 was equilibrated with 25 μl of plasma sample at 37°C. 25 μl of 10.0 NIH U/ml human thrombin was added and incubated for exactly one minute at which time, 50 μl of spectrozyme TH 1 mM was added. The change in absorbance was recorded at 405 nm for one minute. % Inhibition of factor IIa activity was calculated as shown on the next page.

$$\% \text{ Inhibition} = \frac{\text{plasma sample delta A } 405 \text{ nm/min}}{\text{plasma blank delta A } 405 \text{ nm/min}} - 1 \times 100$$

C. In Vitro Studies

1. Baseline Values of NHP, NMP, NRP and NratP

Human plasma obtained from Loyola University Blood Bank (NHP), and plasma obtained from primates (NMP), rabbits (NRP) and rats (NratP) was used to determine the clotting and amidolytic baseline values of these plasma preparations. These experiments were performed in triplicate on separate days.

2. In Vitro Supplementation of Heparin or CY 216 to NHP, NMP, NRP and NratP

Heparin or CY 216 was supplemented in a concentration range of 0 to 10 μg/ml to NHP, NMP, NRP and NratP. APTT, Heptest^R, amidolytic

anti Xa and amidolytic anti IIa assays were performed on these samples.

3. In Vitro Supplementation of Protamine to NHP, NMP, NRP and NratP

Protamine sulfate (10 and 100 μg/ml) was supplemented to NHP, NMP, NRP and NratP. Additional protamine concentrations (1 mg/ml and 10 mg/ml) were supplemented to NHP. APTT, Heptest^{R}, amidolytic anti Xa and amidolytic anti IIa assays were performed on these samples.

4. In Vitro Neutralization of Heparin and CY 216 by Protamine In Pooled Plasma Preparations

Heparin or CY 216 was supplemented to NHP or NMP to obtain a final concentration of 5 μg/ml. Protamine sulfate was added to the heparin (5 μg/ml) and CY 216 (5 μg/ml) solutions to obtain final protamine concentrations of 2.5, 5.0 and 10.0 μg/ml. Anticoagulant activity was determined by the APTT and thrombin time methods. Antiprotease activity was determined by the Heptest^{R}, amidolytic anti Xa and amidolytic anti IIa assays. The experiment was repeated five times on different days using the same pool.

5. In Vitro Neutralization of Heparin and CY 216 by Spermidine In NHP

Heparin or CY 216 was supplemented to NHP to obtain a final concentration of 5 μg/ml. Spermidine was added to the heparin (5 μg/ml) and CY 216 (5 μg\ml) solutions to obtain final spermidine concentrations of 2.5, 5.0 and 10.0 μg/ml. Anticoagulant activity was determined by the APTT and thrombin time. Antiprotease activity was determined by the amidolytic anti Xa and amidolytic anti IIa methods. The experiment was repeated three times on different days using the same pool.

D. Rat Laser-Induced Thrombosis Studies

The in vivo antagonism of heparin and CY 216 by protamine sulfate was studied in a rat model of laser-induced thrombosis. (Breddin et al. 1982; Weichert et al. 1983, 1984, 1986; Racanelli et al. 1989) This model provided a dynamic environment in which platelet activation and subsequent thrombus formation in rat mesenteric vessels can be visualized.

Experiments were performed after anesthetizing the rats with Nembutal (60 mg/kg i.p.). The abdomen were shaved and a small abdominal incision was made. Drugs were injected via the tail vein for intravenous administration and behind the neck for subcutaneous administration. A portion of the rat mesentery was exposed and positioned on an optical stage. In these studies the laser damage was produced by exposing terminal mesenteric venules of 25-30 um diameter to 0.05 Watts of laser energy for 1/30th of a second. Laser damage to the endothelium was visualized through the microscope and antithrombotic effect was calculated in terms of the number of laser injuries required to produce a defined thrombus. Laser injuries were produced in 3 distinct areas of the mesentery per rat. At the end of the experiment blood was drawn from the inferior vena cava and the rat was sacrificed by cardiac incision. The experimental schedule was as follows:

Intravenous Studies:

0	5	20 (minutes)
Inject Heparin or CY 216	Inject Protamine	Produce Laser Injury

Subcutaneous Studies:

0	120	140 (minutes)
Inject Heparin or CY 216	Inject Protamine	Produce Laser Injury

E. Rat Tail Bleeding Studies

A modified version of the rat tail bleeding time (Dejana 1982) was used to determine the hemorrhagic effect of heparin, CY 216 and protamine as well as bleeding time after antagonism of heparin and CY 216 by protamine. The rats were anaesthetized with Ketalar^{R} (40 mg/kg i.m.) and the tail was cleansed with an alcohol swab. Two mm of the distal end of the tail was transected using a scalpel. Blood from the incision site was soaked with a piece of filter paper very carefully so as not to disrupt any hemostatic plug formation. Heparin (2 mg/kg i.v. and 2.5 mg/kg s.c.) and CY 216 (2 mg/kg i.v. and 2.5 mg/kg s.c.) and protamine (1, 2, and 2.5 mg/kg i.v.) were studied in this model.

The experimental schedule was as follows:

Intravenous Studies:

0	5	10 (minutes)
Inject Heparin or CY 216	Inject Protamine	Transect Tail

Subcutaneous Studies:

0	115	120 (minutes)
Inject Heparin or CY 216	Inject Protamine	Transect Tail

F. Rabbit Stasis Thrombosis Studies

The antithrombotic effects of heparin, CY 216 and protamine were studied in a modified stasis thrombosis model (Fareed et al. 1985). White New Zealand male rabbits were anaesthetized with Xylazine (20 mg/kg i.m.) and Ketalar^{R} (80 mg/kg i.m.). If the rabbits showed signs of being too lightly anesthetized during the procedure, additional injections of Ketalar^{R} (25 mg/kg i.m.) were administered. After induction of anaesthesia, the rabbits were weighed, their neck area was shaved and positioned for surgery. Baseline blood samples were drawn by cardiac puncture using a Vacutainer^{R}. A vertical incision in the neck area was made using a scalpel. A blade, forceps and cautery were utilized to surgically isolate the jugular veins from the fascia. The dissection was done carefully so as not to damage or traumatize the vessels and collateral vessels were cauterized. A 2 to 3 cm segment of each jugular vein (including the bifurcation) was isolated and silk sutures were loosely tied around each branch of the jugular vein without interfering with blood circulation. The exposed neck area was kept moist with saline soaked gauze squares.

An intravenous line was set up with a 23 gauge, 3/4 inch butterfly needle in the rabbit marginal ear vein. Heparin or CY 216 was injected and allowed to circulate for 5 minutes at which time, blood for ex vivo analysis was drawn by cardiac puncture. Immediately after the blood draw, PCC (25 U/kg) was administered through the marginal ear vein followed by RVV (0.1 U/kg) which was allowed to circulate for exactly 20 seconds. At this time the isolated jugular vein segments were ligated and stasis produced. A schematic diagram of the isolated

jugular veins ar shown in Appendix VIII. Blood was drawn for ex vivo analysis, five minutes post PCC/RVV injection. Exactly 10 minutes after stasis time, the isolated jugular segments were removed and placed in a petri dish with saline. The blood clots were visually examined and graded according to the following method. In this system + 0 represented blood with no evidence of clotting, + 1 represents some minute clots however mostly blood, + 2 represents a large amount of small clots, +3 represents large clots with some free red blood cells, + 4 represents a fully formed casted clot with no blood. A schematic diagram of the clot grading system which was used is shown in Appendix IX. Rabbits were euthanized with Beuthanasia^{R} (15 mg/kg i.v.). The experimental schedule was as follows:

Intravenous Studies: ↓ = blood draw

0	5	10	15	20 (min)
Draw Blood Then Inject Heparin or CY 216	Inject Protamine	Draw Blood Inject PCC/RVV Then Ligate Vessel	Draw Blood	Remove Vessels and Evaluate Clots

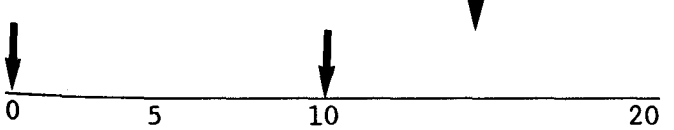
Subcutaneous Studies: ↓ = blood draw

0	245	255	265	275 (min)
Draw Blood Then Inject Heparin or CY 216	Inject Protamine	Draw Blood Inject PCC/RVV Then Ligate Vessel	Draw Blood	Remove Vessels and Evaluate Clots

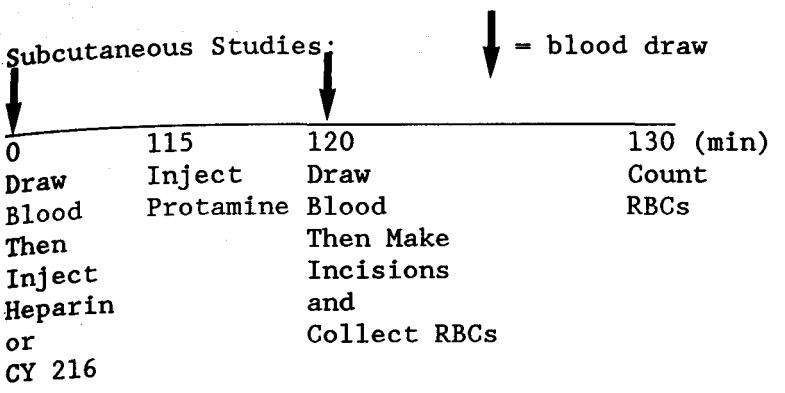
G. Rabbit Ear Blood Loss Studies

A modified version of the rabbit ear blood loss model (Cade et. al. 1984), was used to compare the hemorrhagic potential of heparin, CY 216, and protamine. The rabbits were anesthetized with Xylazine (10 mg/kg i.m.) and Ketalar^{R} (40 mg/kg i.m.). After induction of anaesthesia, an intravenous line was set up with a 23 gauge, 3/4 inch butterfly needle inserted in the large marginal ear vein through which heparin, CY 216 and protamine were administered. The rabbit ear was immersed in a saline bath at 37^{o}C and an area free of major blood vessels was selected. Using a scalpel, five uniform incisions of full thickness were made in the area which was free of major blood vessels as shown in the Appendix X. Immediately after the incisions were made, the rabbit ear was immersed in the saline bath and the red blood cells were collected for 10 minutes. The saline was collected and the red cells were counted using a Hycel^{R} cell counter. Heparin (2 mg/kg i.v.) and CY 216 (2 mg/kg i.v.) and protamine (1, 2 and 3 mg/kg i.v.) were studied. Heparin (1 mg/kg s.c.) and CY 216 (1 mg/kg s.c.) were also studied. The protocol used to study blood loss is in this model is shown below. After the experiment the rabbits were returned to their cages and used for the stasis thrombosis experiments, no less than one week later (Emanuele 1987; Boneu 1987).

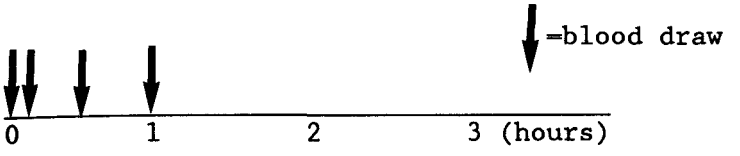
Intravenous Studies: ↓ = blood draw



0	5	10	20 (min)
Draw Blood Then Inject Heparin or CY 216	Inject Protamine	Draw Blood Then Make Incisions and Collect RBCs	Count RBCs



Protamine sulfate was administered at a dose of 2.1 mg/kg i.v. and blood was drawn as shown below:



The plasma samples were centrifuged and frozen at - 70° C. The pharmacodynamics of protamine were assayed indirectly by supplementing the plasma of the primates which received protamine (2.1 mg/kg i.v.) with heparin in order to obtain a final heparin concentration of 2.5 μg/ml. APTT, thrombin time, Heptest^{R}, amidolytic anti Xa and amidolytic anti IIa assays were performed. The ability of protamine in the plasma sample to neutralize the exogenously added heparin was indicative of the protamine concentration in the plasma. Additional in vitro studies were carried out where APTT, thrombin time, Heptest^{R}, amidolytic anti Xa and amidolytic anti IIa assays were performed on the ex vivo plasma samples after protamine administration.

H. Time Course of the Pharmacodynamic Effects of Heparin and CY 216 in Primates

The time course of pharmacodynamic activity of heparin, CY 216, and protamine sulfate was studied using a primate model (Fareed 1985). In addition, the time course of the neutralization of heparin and CY 216 by protamine was studied.

Baseline blood samples were drawn from all of the primates prior to beginning the study. Chemistry, coagulation and hematological profiles were determined by the Loyola University Medical Center department of clinical chemistry as shown in Appendix XI. Of the 12 primates that were profiled, two were excluded from the study for health reasons. The remaining 10 primates were divided into 2 groups consisting of 5 primates each. These two groups were used alternatively for the duration of the study. Each primate was only used once weekly which provided adequate time for drug clearance (Emanuele 1987). A one month rest period was given to the primates between the intravenous and subcutaneous time course studies. The primates were grouped in a random manner as shown below.

Group I	Group II
M73	M70
M74	M75
M76	M2722
M2730	M3630
M3064	M4777

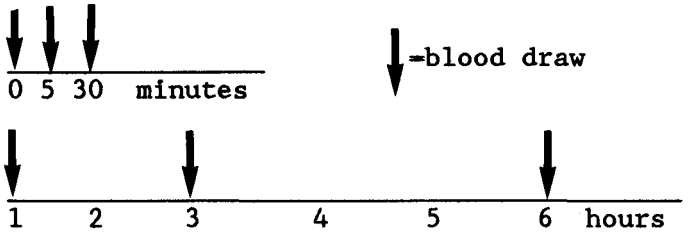
All pharmacodynamic time course experiments began at the same time of the day (9:00 to 9:30 a.m.). Prior to blood sampling the

animals were anesthetized with Ketalar^{R} (10 mg/kg i.m.). Approximately 10 minutes after injection, the primates were removed from their cages and baseline blood samples were drawn from the popliteal vein. All drugs were injected through syringes incorporating sterile, pyrogen free 0.2 um Nalgene filters (Nalge Co. Rochester NY). Primates were returned to their cages and monitored as they recovered from their anesthetic. Studies have shown that repeated administration of Ketalar^{R} does not have an effect on the primate coagulation profile (Fareed et al. 1985).

1. Heparin and CY 216 Pharmacodynamics

a. Intravenous Studies

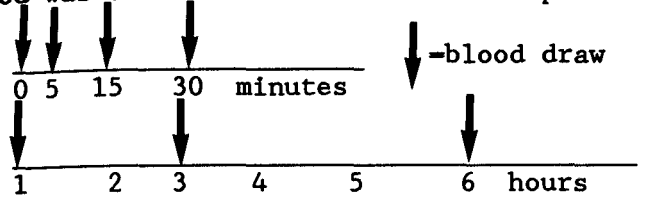
After 0 hour blood samples were drawn from each primate, heparin or CY 216 were administered at doses of 0.35 mg/kg i.v. and 0.7 mg/kg i.v. Blood samples (2.7 ml) were collected according to methods previously described in this section, into siliconized glass tubes containing 0.3 ml of 3.8 % sodium citrate and centrifuged to obtain plasma. The experimental schedule for the intravenous studies is shown below. The number in parentheses refers to primate group numbers.



1. Heparin 0.35 mg/kg i.v. (I)
2. Heparin 0.70 mg/kg i.v. (II)
3. CY 216 0.35 mg/kg i.v. (II)
4. CY 216 0.70 mg/kg i.v. (I)

The clotting tests APTT, thrombin time and Heptest^{R} were performed on fresh samples and plasma was frozen for subsequent in vitro analysis.

For the neutralization studies heparin (0.7 mg/kg i.v.) or CY 216 (0.7 mg/kg i.v.) was administered and protamine was given 5 minutes after heparin administration. Blood samples were drawn as shown below. Blood was drawn at time 0 and then heparin or CY 216 was injected.

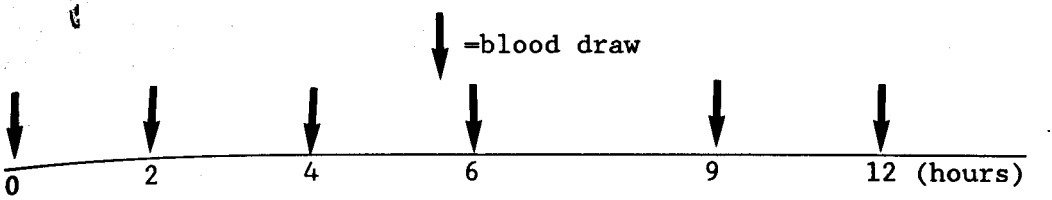


The above schedule was used for the following studies. The number in parentheses represents the primate group number.

1. Heparin 0.7 mg/kg i.v. w/ Protamine Sulfate 0.7 mg/kg i.v. (I)
2. Heparin 0.7 mg/kg i.v. w/ Protamine Sulfate 1.4 mg/kg i.v.(II)
3. Heparin 0.7 mg/kg i.v. w/ Protamine Sulfate 2.1 mg/kg i.v.(II)
4. CY 216 0.7 mg/kg i.v. w/ Protamine Sulfate 0.7 mg/kg i.v. (I)
5. CY 216 0.7 mg/kg i.v. w/ Protamine Sulfate 1.4 mg/kg i.v.(II)
6. CY 216 0.7 mg/kg i.v. w/ Protamine Sulfate 2.1 mg/kg i.v. (I)

b. Subcutaneous Studies

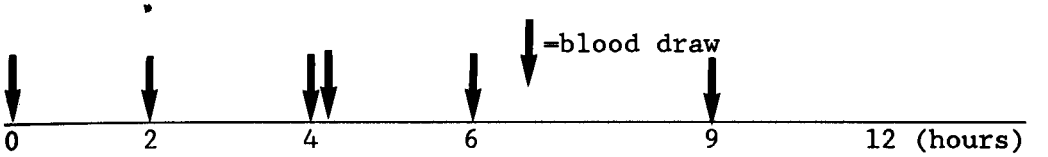
Heparin and CY 216 were administered at 0.7 mg/kg s.c. by a single bolus injection to a site in the lower abdomen for all primates except M4777. This primate received a divided dose in order to keep the volume injected less than 1 ml. Blood samples were drawn as shown below. Blood was drawn at time 0 and then heparin or CY 216 was injected.



The above schedule was used for the following studies. The number in parentheses represents the primate group number.

1. Heparin 0.7 mg/kg s.c. (I)
2. CY 216 0.7 mg/kg s.c. (II)

Blood was drawn by the following schedule for primates which received protamine in addition to heparin (0.7 mg/kg s.c.) or CY 216 (0.7 mg/kg s.c.). Protamine was administered immediately after the 4 hour blood draw.



1. Heparin 0.7 mg/kg s.c. w/ Protamine Sulfate 0.7 mg/kg i.v. (I)
2. CY 216 0.7 mg/kg s.c. w/ Protamine Sulfate 0.7 mg/kg i.v. (I)
3. CY 216 0.7 mg/kg s.c. w/ Protamine Sulfate 0.7 mg/kg i.v. (II)

Studies on the neutralization of heparin (0.7 mg/kg s.c.) or CY 216 (0.7 mg/kg s.c.) by protamine were carried out at the following doses of protamine sulfate. The number in parentheses refers to the primate group number.

1. Heparin 0.7 mg/kg s.c. w/ Protamine Sulfate 0.7 mg/kg i.v.(II)
2. CY 216 0.7 mg/kg s.c. w/ Protamine Sulfate 0.7 mg/kg i.v. (I)
3. CY 216 0.7 mg/kg s.c. w/ Protamine Sulfate 1.4 mg/kg i.v.(II)

2. Calculation of Pharmacodynamic Time Course Parameters

Pharmacodynamic time course parameters for the intravenous and subcutaneous studies were determined from concentrations (μg/ml)

obtained from calibration curves of heparin or CY 216 supplemented to NMP as determined by the Heptest^{R} and amidolytic anti Xa assays. These two assays were chosen to calculate the pharmacodynamic time course parameters since they primarily measure anti Xa activity. This activity is commonly used to monitor low molecular weight heparin therapy. In addition, these assays demonstrated the greatest linearity over the concentrations studied and only the linear portion of the calibration curves were used. If a primate sample was determined to be out of range of the curve, it was diluted 1:5 in normal monkey plasma, read off the curve and multiplied by the dilution factor (Sie et al. 1989). The calibration curves of heparin and CY 216 supplemented in NMP are shown in the Appendix XII.

Pharmacodynamic time course parameters were determined using noncompartmental methods as described by Gibaldi (1984). The pharmacodynamics of intravenously or subcutaneously administered heparin and CY 216 were characterized in terms of area under the concentration curve (AUC), area under the moment curve (AUMC), mean residence time (MRT), plasma clearance (Clp) and apparent volume of distribution (Vd). Area under the curve and AUMC were calculated using PCNONLIN software (Statistical Consultants, Lexington, KY).

The mean residence time (MRT) is the time when 63.2% of an intravenous dose has been eliminated. The concept is similar to the biologic half life when 50% of the dose has been eliminated. MRT is calculated by the ratio of the AUMC to AUC.

$$MRT = 60(min) \times \frac{AUMC}{AUC}$$

plasma clearance was determined by the following formula:

$$Clp = \frac{DOSE}{AUC} \times \frac{1}{60(min)}$$

Apparent volume of distribution was determined by the following formula:

$$Vd = \frac{dose \times AUMC}{(AUC)(AUC)} \times \frac{1\ liter}{1000\ ml}$$

Bioavailability after subcutaneous administration was calculated by the following formula:

$$Bioavailability = \frac{mean\ AUC\ (s.c.\ dose)}{mean\ AUC\ (i.v.\ dose)}$$

Protamine Neutralization Index (PNI) (Bjornsson personal communication) was calculated by the following formula:

$$PNI = 1 - \frac{post\ protamine\ AUC}{(mean)\ pre\ protamine\ AUC}$$

In order to calculate PNI values for the primate studies, it was necessary to use the mean value for the protamine neutralization, since the experiments were performed using groups and not all of the primates were used for all of the studies.

For the amidolytic assays, % neutralization was calculated by the following formula:

$$\%\ Neutralization = \frac{post\ protamine\ \mu g/ml}{pre\ protamine\ \mu g/ml} - 1$$

% N were calculated at 30 minutes for intravenous studies and at 245 minutes for the subcutaneous studies for the primates. In the rabbit studies, % N was calculated after 10 minutes for the intravenous studies and after 120 minutes for the subcutaneous studies. % N was

not calculated for the APTT, thrombin time and Heptest^R data since many values were outside of the linear range of the assay.

I. Statistical Analysis

1. Data Presentation

The experimental data was compiled as mean ± S.E. (standard error of the mean). The sample mean was the average of the measurements within each experimental group. The standard error of the mean (S.E.) was reported along with the mean in order to provide an indication of the precision of estimation of the population mean. The S.E. was also used in order to facilitate comparison of the means of one group to another. The S.E. was calculated by dividing the standard deviation (S.D.) by the square root of the number in the experimental population.

$$S.E. = \frac{S.D.}{n^{1/2}}$$

The data in the figures was represented as mean ± S.E. Error bars were drawn using the Sigmaplot^R software and the error bars are not shown if they are not larger than the size of the symbol in the figure.

2. Statistical Tests

Statistical tests performed on the experimental data include: Students t-test (unpaired), one way ANOVA, two way ANOVA, Students-Newman Keuls multiple comparison test, Kruskal Wallis nonparametric ANOVA, nonparametric multiple comparison test and linear regression. All tests with the exception of the Kruskall-Wallis nonparametric ANOVA and the linear regression tests were performed utilizing Systat. The linear regressions were performed utilizing Sigmaplot and the non-parametric tests were calculated manually. All test statistics were

calculated at a significance level of .05. Critical values for t, F, H, and q distributions were obtained from Zar (1984).

a. Student's t test

The student's t test was used to compare the means from the heparin group to the means from the CY 216 group in the primate pharmacodynamic time course data. Two tailed t tests were used for all of the analyses which indicates the mean value obtained could be greater or less than the mean value from the other group.

b. Analysis of Variance

The one way ANOVA was used to compare the effect of a single factor on a single variable although different factor levels exist. The one way ANOVA was used to compare several group means after a treatment. The test for the equality of means is a one-sided variance-ratio test as shown below:

$$F = \frac{\text{group mean square}}{\text{error mean square.}}$$

The critical value for this test is $F_{\text{alpha } (1),(k-1)(N-k)}$ where N = total number of data points and k = number of groups. If the calculated F is at least as large as the critical value, then H_o is rejected. The ANOVA does not determine where the differences between means exist, if indeed this test concludes that a significant difference does exist between the means. If the ANOVA revealed a significant difference, the Student-Newman-Keuls multiple comparison test was used to determine the location of the differences between the means.

The two way ANOVA simultaneously analyzes the effect of more than one factor on a group mean. This test considers the effects of two

factors independently of each other. For a two way ANOVA, three F values are obtained. F values for Factor A, Factor B and the interaction between Factors A and B are obtained. An interaction between two factors indicates that the effect of one factor is not independent of the presence of a particular level of the other factor.

c. Student-Newman-Keuls Test

The Student-Newman-Keuls test was applied in addition to the ANOVA to determine which means were statistically different from the other. This multiple comparison was selected since it is more powerful and widely accepted than the other multiple comparison tests (Zar 1984). The test statistic q is calculated and the critical value is $q_{alpha,\ DF,\ p}$ where p is the number of means in the range of means being tested.

$$q = \frac{X_B - X_A}{S.E.}$$

If the number of groups (k) were equal, the S.E. was calculated as

$$S.E. = (s^2/n)^{1/2}$$

where s^2 = error mean square.

If the number within each group were not equal, the S.E. was calculated as

$$S.E. = (s^2/2\ (1/n_a + 1/n_b))^{1/2}$$

For S.E. n= number of data points in each level, v= with in cells degrees of freedom. Newman-Keuls multiple comparison tests were performed following 2 way ANOVAs. The comparisons were made between the levels of a fixed effects factor having more than two levels.

d. Kruskall-Wallis Test

The Kruskal Wallis nonparametric ANOVA was used to test for differences between the clot scores obtained in the stasis thrombosis model. The Kruskal Wallis test statistic H was calculated as

$$H = \frac{12}{N(N+1)n_i} R_i^2 - 3(N + 1)$$

where N = total number of observations in all groups, n_i = number of observations in group I and R_i = sum of ranks of n_i observations in a group.

e. Nonparametric Multiple Comparison Test

Nonparametric multiple comparison tests were used when the nonparametric Kruskal-Wallis test was applied and significant difference between means was obtained. Nonparametric comparisons are performed in a similar manner to the Tukey test, however the rank sums are used instead of means. The S.E. was calculated as

$$S.E. = \frac{(n(nk)(nk+1)}{12}$$

f. Simple Regression

Simple regression analysis was performed to establish the relationship between ex vivo activity and either blood loss or % inhibition of thrombosis. The coefficient of determination R^2 was computed using Sigmaplot^R solftware and the r value was reported. The average number of data points used to calculate each r value was 10.

Chapter IV

RESULTS

A. In Vitro Studies

1. Molecular Weight Profiles of Heparin, CY 216 and Protamine

The molecular weight profile for heparin as determined by a high pressure liquid chromatographic (HPLC) method as previously described by Losito (1981) is shown in Appendix III. Heparin exhibited a polydisperse molecular weight profile with a mean molecular weight of 11,000 daltons.

The molecular weight profile for CY 216 as determined by a high pressure liquid chromatographic (HPLC) method using a UV detector is shown in Appendix V. CY 216 exhibited a polydisperse molecular weight profile with a mean molecular weight of 5,200 daltons.

The molecular weight profile for protamine as determined by a high pressure liquid chromatographic (HPLC) method using a UV detector is shown in Appendix VII. Protamine exhibited a polydisperse molecular weight profile with a mean molecular weight of 4,000 daltons.

The molecular weight profiles for heparin and CY 216 were obtained in collaboration with Institut Choay.

2. NMR Spectra of Heparin, CY 216 and Protamine

Proton NMR spectra were also used to profile heparin, CY 216, and protamine. The ^{1}H NMR spectras were obtained in collaboration of Professor Casu of Institute Ronzoni, Milan Italy. The spectra were measured with a Bruker HX-270 spectrometer operating at 300 MHz.

Chemical shifts were measured with reference to internal TSP. The ^1H NMR spectrum (300 MHz, D_2O) of heparin is shown in Appendix XIII. This spectrum shows the regular regions consisting of $GlcNSO_3$ and IdoA-2S residues of the regular sequences of heparin. This data is consistent with the proposed structure of heparin. Appendix XIV shows a comparable spectrum of CY 216 which was obtained under identical conditions as the heparin spectrum. Differences between the spectral characteristics of heparin and CY 216 were observed in the region of 3-4.5 ppm. Furthermore the spectra demonstrated the presence of anhydromannose residues which are present at the terminal end groups of some low molecular weight heparins. Appendix XV shows the NMR spectrum of protamine and a complex formed between heparin and protamine. The spectrum obtained for protamine was markedly different than the spectra obtained for the heparins. Preliminary analysis of this heparin-protamine complex data demonstrates a chemical shift in the 3.4-3.6 ppm region (see box) as shown in Appendix XV.

3. Baseline Values of NHP, NMP, NRP and NratP in the Clotting and Amidolytic Assays.

Human pooled plasma was obtained from Loyola University Blood Bank (NHP), and pooled plasma obtained from primates (NMP), rabbits (NRP) and rats (NratP) were used in this study. Baseline values of these plasma preparations were determined in the clotting and amidolytic assays and statistical analysis was performed by a one way ANOVA. The results are shown in Table 1. Variations in the baseline APTT values for NHP, NMP and NratP were observed. The baseline values for NRP and NratP were significantly different ($p<.05$) from NHP. The

baseline clotting values obtained for NHP and NMP were similar as determined by the thrombin time, however the baseline values for NRP and NratP were significantly longer ($p<.05$) as compared to NHP. The values were similar for NHP and NMP as determined by the Heptest^R (15.5 and 20.5 seconds) respectively, however, the NratP baseline value was significantly longer ($p<.05$) than NHP at 56.6 seconds. The baseline chromogenicity of NMP as determined by the amidolytic anti Xa and amidolytic anti IIa assays were slightly higher than for the other plasma samples studied. However, % inhibition of amidolytic factor X_a or amidolytic factor II_a activity was calculated as a ratio. Any variations between the absorbances of the plasma samples were minimized after the calculation of % inhibition was performed. Control values for the amidolytic assays were designated as 0% inhibition.

4. In Vitro Supplementation of Protamine Sulfate to NHP, NMP, NRP and NratP

The in vitro results of protamine sulfate supplementation to the various plasma preparations in a concentration range of 0 - 100 μg/ml are shown in Table 2. Additional concentrations of protamine (1 and 10 mg/ml) were supplemented to NHP in order to demonstrate the effects of high concentrations of protamine on human plasma. These concentrations of protamine were larger than doses which would be administered to a patient after cardiopulmonary bypass.

NRP was more sensitive to the effects of protamine in the in vitro assays than the other plasma preparations. NHP was not significantly prolonged, (as determined by a one way ANOVA), by protamine sulfate until the protamine concentration reached 1 mg/ml. Protamine

(100 μg/ml) did not have any effect on the absorbance values as determined by the amidolytic anti Xa and amidolytic anti IIa methods in any of the plasma systems as shown in Table 2.

5. In Vitro Supplementation of Heparin and CY 216 to NHP, NMP, NRP and NratP

Heparin or CY 216 was supplemented to NHP, NMP, NRP and NratP in a concentration range of 0 to 10 μg/ml. Plasma preparations were obtained as previously described in methods. The experiment was performed on 3 separate days using aliquots of the same frozen pooled plasma preparations. APTT, Heptest^{R}, amidolytic anti Xa and amidolytic anti IIa assays were performed on these samples.

The anticoagulant effects of heparin supplemented to the various plasma systems as determined by the APTT are shown in Figure 1. In a concentration range of 0-10 μg/ml, heparin produced a similar dose dependant prolongation of NHP and NMP. In addition dose dependant increases in the APTT were observed for NRP and NratP. The responses in NRP were stronger than for NHP, whereas NratP exhibited weaker responses.

The anticoagulant effects of CY 216 supplemented to the plasma systems as determined by the APTT are shown in Figure 2. NRP was also prolonged to the same degree although the curve was shifted along the y axis due to variations in baseline values for NRP. NratP was less sensitive to the effects of CY 216 than the other plasma preparations as determined by the APTT.

The antiprotease activity of heparin in various plasma systems as determined by the Heptest^{R} is shown in Figure 3. At the lower con-

centrations, heparin prolonged NHP, NMP and NratP to the same extent, however, a divergence in this activity was seen at concentrations > 5 μg/ml. The Heptest^{R} was very sensitive to the effects of heparin (5 μg/ml) supplemented to NRP and a prolongation of > 300 seconds was observed.

The antiprotease activity of CY 216 in the various plasma systems as determined by the Heptest^{R} is shown in Figure 4. CY 216 prolonged NHP and NMP in a similar manner while CY 216 prolonged the Heptest^{R} to a slightly lesser degree as compared to heparin. NRP and NratP were also prolonged to the same extent, although the NratP baseline was elevated as compared to the other plasmas.

The amidolytic anti Xa activity of heparin supplemented to various plasma systems is shown in Figure 5. In a concentration range of 0-10 μg/ml, heparin produced a similar dose dependant increase in the inhibition of amidolytic anti Xa activity of NHP and NMP. NRP and NratP also produced a dose dependant inhibition, however it was somewhat weaker than the response of NHP.

The amidolytic anti Xa activity of CY 216 supplemented to various plasma systems is shown in Figure 6. CY 216 in a concentration range of 0-10 μg/ml, produced a similar dose dependant increase in the inhibition of amidolytic anti Xa activity of NHP and NMP, however the assay exhibited the most sensitivity to the antiprotease effects of CY 216 supplemented to NHP. NRP and NratP also produced dose dependant inhibition, however these responses were somewhat weaker than the response of NHP. NRP was less sensitive to the antiprotease effects of CY 216 than the other plasma preparations.

The amidolytic anti IIa activity of heparin supplemented to various plasma systems is shown in Figure 7. In a concentration range of 0-1.25 μg/ml, heparin produced a similar dose dependant increase in the inhibition of amidolytic anti IIa activity of NHP and NMP. However NMP reached the upper limit of the linear range of this amidolytic anti IIa assay at a lower concentration than did the NHP. NRP and NratP also produced similar dose dependant inhibition, however it was somewhat weaker than the response of NHP.

The amidolytic anti IIa activity of CY 216 supplemented to various plasma systems is shown in Figure 8. In a concentration range of 0-10 μg/ml, CY 216 produced a similar dose dependant increase in the inhibition of amidolytic anti IIa activity of NHP, NRP and NratP, however, the assay exhibited the greatest sensitivity to the anti-protease effects of CY 216 supplemented to NMP.

The numerical values for the data plotted in Figures 1-8 are shown in Appendix XXIII. A comparison of the amidolytic anti Xa and anti IIa activities of heparin is shown in Appendix XIIe. A comparison of the amidolytic anti Xa and anti IIa activities of CY 216 is shown in Appendix XIIf.

6. In Vitro Protamine Neutralization of Heparin and CY 216 in NHP

The in vitro protamine neutralization of heparin and CY 216 supplemented to NHP or NMP as determined by the APTT is shown in Figure 9. At a concentration of 5 μg/ml, CY 216 only slightly prolonged the APTT, while heparin prolonged the APTT to > 150 seconds. A progressive degree of neutralization of heparin by protamine (0-10 μg/ml) was

noted. At equigravimetric concentrations of heparin and protamine (5 μg/ml) a complete neutralization of APTT activity was seen.

The in vitro protamine neutralization of heparin and CY 216 in NHP or NMP as determined by the thrombin time assay is shown in Figure 10. Heparin (5 μg/ml) prolonged the thrombin time to a greater extent than CY 216 (5 μg/ml). Complete neutralization of the thrombin time activity of heparin was seen at a gravimetric protamine to heparin ratio of 1:1 while the thrombin time activity of CY 216 was completely neutralized at a gravimetric protamine to CY 216 ratio of 1:2.

The in vitro protamine neutralization of heparin and CY 216 in NHP or NMP as determined by the Heptest^{R} is shown in Figure 11. Heparin (5 μg/ml) prolonged the Heptest^{R} to a greater extent than CY 216 (5 μg/ml). Almost complete neutralization of the Heptest^{R} activity was seen at a gravimetric protamine to heparin ratio of 2:1. Only a slight degree of neutralization of Heptest^{R} activity was seen at a gravimetric protamine to CY 216 ratio of 2:1.

The in vitro protamine neutralization of heparin and CY 216 in NHP or NMP as determined by the amidolytic anti Xa assay is shown in Figure 12. Heparin (5 μg/ml) inhibited the amidolytic activity of factor Xa to a greater extent than CY 216 (5 μg/ml). Almost complete neutralization of the amidolytic anti Xa activity of heparin was seen at a protamine to heparin ratio of 1:1. Only a slight degree of neutralization of amidolytic anti Xa activity was seen at a gravimetric protamine to CY 216 ratio of 2:1.

The in vitro protamine neutralization of heparin and CY 216 in NHP or NMP as determined by the anti IIa assay is shown in Figure 13.

Heparin (5 μg/ml) inhibited amidolytic factor IIa activity to a greater extent than CY 216 (5 μg/ml). Almost complete neutralization of the anti IIa activity of heparin and CY 216 was seen at a gravimetric protamine to heparin (or CY 216) ratio of 1:1.

The numerical values for the data plotted in Figures 9-13 are shown in Appendices XXIIIi and XXIIIj.

7. In Vitro Neutralization of Heparin and CY 216 By Spermidine In NHP

The in vitro neutralization of heparin by spermidine in NHP is shown in Table 3. Supplementation of spermidine (10 μg/ml) had no effect on the clotting or amidolytic assays. Heparin (5 μg/ml) prolonged the APTT and thrombin time assays to > 150 while the Heptest^{R} was prolonged to a lesser degree by heparin (5 μg/ml). Heparin (5 μg/ml) exhibited substantial inhibition of the amidolytic anti Xa and amidolytic anti IIa activity. Spermidine was a less potent antagonist of the anticoagulant and antiprotease activities of heparin than protamine. The APTT and thrombin time activity of heparin (5.0 μg/ml) remained prolonged to > 150 seconds even after supplementation of a two fold gravimetric amount of spermidine. The anti IIa activity of heparin was neutralized to a greater degree than the other activities. In contrast, complete neutralization of the anticoagulant and antiprotease activity of heparin was observed at two fold gravimetric concentrations of protamine or less.

The in vitro neutralization of CY 216 by spermidine in NHP is shown in Table 4. CY 216 (5.0 μg/ml) prolonged the APTT to a lesser degree than heparin (5.0 μg/ml). No neutralization of the APTT or

Heptest^R activity was seen by spermidine (2.5, 5.0, or 10 μg/ml) while complete neutralization of the APTT activity was noted at a spermidine concentration of 1 mg/ml. In contrast, Heptest^R activity was only partially neutralized by spermidine (1 mg/ml). The amidolytic anti Xa activity of CY 216 was less neutralizeable than the amidolytic anti IIa activity of CY 216. It is interesting to note that the amidolytic activity was neutralized to a greater degree than the thrombin time clotting activity which suggests that there may be specific structural requirements for the neutralization of the different activities of heparin as determined by the laboratory assays.

B. In Vivo Studies

1. Rat Model of Laser-Induced Thrombosis

a. Antithrombotic Effects of Protamine

The effects of protamine sulfate on laser induced thrombosis in rats is shown in Figure 14. An increase in number of laser injuries required to produce a defined thrombus is related to the antithrombotic effect of the drug. Protamine (0.74 mg/kg i.v.) increased the number of laser injuries however this increase was not significant ($p>.20$) as determined by an unpaired student t test.

b. Intravenous Studies

Protamine antagonism of heparin (1 mg/kg i.v.) in the laser induced model of thrombosis is shown in Figure 15. Heparin (1 mg/kg i.v.) significantly ($p<.05$), (as determined by a one way ANOVA) increased the number of laser injuries needed to produce a defined thrombus. This increase correlates to antithrombotic effect in this model. Protamine (0.32 mg/kg i.v.) did not reduce the number of laser

injuries needed to produce a defined thrombus and therefore did not antagonize the antithrombotic activity of heparin (1 mg/kg i.v.). A protamine concentration of (0.64 mg/kg i.v.) was needed to sufficiently antagonize these antithrombotic effects.

Protamine antagonism of CY 216 (1 mg/kg i.v.) in the laser induced model of thrombosis is shown in Figure 16. CY 216 (1 mg/kg i.v.) significantly ($p<.05$), (as determined by a one way ANOVA) increased the number of laser injuries required to produce a defined thrombus in the laser model of thrombosis. A protamine concentration of (0.74 mg/kg i.v.) completely antagonized the antithrombotic effects of CY 216.

c. Subcutaneous Studies

The effect of subcutaneously administered heparin and CY 216 is shown in Figure 17. Both heparin and CY 216 at doses of (10 mg/kg s.c.) significantly ($p < .05$), (as determined by a one way ANOVA), increased the number of laser injuries required to produce a defined thrombus.

The numerical values for the data plotted in Figures 14-17 are shown in Appendix XXIVa.

2. Rabbit Stasis Thrombosis Model

A rabbit stasis thrombosis model was used to evaluate the antithrombotic effects of heparin and CY 216 and the comparative protamine neutralization profiles of these drugs. Ex vivo blood samples were analyzed to determine the ex vivo levels of the various activities at the time thrombosis was induced. Prothrombin complex concentrate and Russel's viper venom were administered to rabbits as a

thrombogenic challenge and clots were graded as described in the methods section. Antithrombotic effects were studied after intravenous and subcutaneous administration of heparin or CY 216 in addition to protamine sulfate.

a. Protamine Sulfate

The antithrombotic activity of protamine sulfate in a rabbit stasis thrombosis model is shown in Figure 18. Concentrations of up to 500 μg/kg i.v. did not have any significant ($p>.10$), (as determined by a Kruskall Wallis test), inhibitory effect upon thrombus formation in this model.

The APTT, thrombin time, Heptest^R, amidolytic anti Xa and amidolytic anti IIa results of rabbits administered protamine sulfate (100 and 500 μg/kg i.v.) are shown in Table 5. Protamine sulfate did not exhibit any significant effects ($p>.20$), (as determined by an unpaired t test), upon the clotting tests or amidolytic at the doses of protamine which were used during the stasis thrombosis experiments.

b. Heparin

The antithrombotic activity of heparin (25, 50 and 100 μg/kg i.v.) is shown in Figure 19. Heparin produced significant ($p<.05$), (as determined by a Kruskall Wallis test), dose dependant inhibition of clot formation at the three doses studied.

The APTT, thrombin time and Heptest^R results of rabbits administered heparin (25, 50 and 100 μg/kg i.v.) as demonstrated by the APTT, thrombin time and Heptest^R are shown in Table 6. Heparin (100 μg/kg i.v.) produced a significant ($p<.05$), (as determined by a one way ANOVA) prolongation of the APTT and Heptest^R. Thrombin time was more

sensitive to the effects of heparin than the other clotting tests. A significant elevation ($p<.05$) was observed for heparin (50 μg/kg i.v.). Statistical analysis was not performed on the heparin (100 μg/kg i.v.) thrombin time data since an indefinite number (> 150 seconds) was obtained as a result.

The ex vivo amidolytic anti Xa and amidolytic anti IIa activity of rabbits administered heparin (25, 50 and 100 μg/kg i.v.) is shown in Figure 20. Heparin produced an inhibition of the amidolytic action of factors Xa and IIa in a dose dependant manner. Significant ($p<.05$) anti Xa activity was seen for heparin (100 μg/kg i.v.) and significant anti IIa activity was seen for heparin (50 μg/kg i.v. and 100 μg/kg i.v.) as determined by a two way ANOVA.

Correlation coefficients were calculated to determine the extent of the relationship between the circulating ex vivo pharmacodynamic activity of intravenously administered heparin and % inhibition of thrombosis in a stasis thrombosis model. Poor correlations were obtained for all of the tests. Anti IIa had the best correlation coefficient ($r=.552$). The other r values are as follows: APTT=.002, thrombin time =.352, Heptest^R =.144, anti IIa =.515.

c. CY 216

The antithrombotic activity of CY 216 (25, 50 and 100 μg/kg i.v.) is shown in Figure 21. CY 216 produced a dose dependant inhibition of thrombosis while CY 216 (100 μg/kg i.v.) significantly ($p<.05$) inhibited clot formation as compared to the control group as determined by a Kruskall Wallis test.

The APTT, thrombin time and Heptest^R results of rabbits administered CY 216 (25, 50 and 100 μg/kg i.v.) is shown in Table 7. CY 216 (25 μg/kg i.v.) did not have a significant effect upon the clotting tests. CY 216 (50 μg/kg i.v.) significantly ($p<.05$) prolonged the thrombin time while CY 216 (100 μg/kg) significantly ($p<.05$) prolonged the thrombin time and Heptest^R as determined by a one way ANOVA.

The ex vivo anti Xa and anti IIa activity of rabbits administered CY 216 (25, 50 and 100 μg/kg i.v.) is shown in Figure 22. Minimal inhibition (<10%) of factor Xa and factor IIa was shown for CY 216.

Correlation coefficients were calculated to determine the relationship between the circulating ex vivo pharmacodynamic activity of intravenously administered CY 216 and % inhibition of thrombosis in a stasis thrombosis model. Poor correlations were obtained for all of the tests. Heptest^R had the best correlation coefficient (r=.806). The other r values are as follows: APTT=.152, thrombin time =.570, anti Xa =.306 and anti IIa =.311.

d. Protamine Antagonism of the Effects of Heparin and CY 216

1. Intravenous Studies

The clot scores obtained after protamine neutralization of either heparin (100 μg/kg i.v.) or CY 216 (100 μg/kg i.v.) is shown in Figure 23. Protamine completely antagonized the antithrombotic activity of heparin (100 μg/kg i.v.) at equigravimetric amounts of protamine and heparin. The antithrombotic activity of CY 216 (100 μg/kg i.v.), however, was not completely antagonized even at a gravimetric ratio protamine to heparin ratio of 2:1. This residual antithrombotic

activity which remained after protamine was significant ($p<.05$) as compared to the control group as determined by a two way ANOVA.

The coagulation parameters of rabbits administered intravenous heparin or CY 216 and protamine as determined by the APTT, thrombin time and Heptest^R are shown in Table 8. Protamine (200 μg/kg i.v.) completely neutralized the thrombin time and Heptest^R activity of heparin (100 μg/kg i.v.) and CY 216 (100 μg/kg i.v.). The Heptest^R activity of heparin was neutralized to a greater degree than the Heptest^R activity of CY 216.

The ex vivo anti Xa activity after protamine neutralization of heparin or CY 216 in rabbits is shown in Figure 24. Heparin exhibited a significant ($p<.05$) degree of inhibition of amidolytic factor Xa activity which was completely neutralized by protamine. CY 216 inhibited factor Xa by approximately 10 % while only a slight degree of neutralization of the anti Xa activity of CY 216 was seen.

The ex vivo anti IIa activity after protamine neutralization of heparin or CY 216 in rabbits is shown in Figure 25. Heparin exhibited significant ($p<.05$), (as determined by a two way ANOVA), inhibition of amidolytic anti IIa activity which was completely neutralized by protamine sulfate. CY 216 administration did not have any effect on this activity.

Poor correlation coefficients were obtained for residual activity after heparin neutralization and clot score ($r=.246$ for APTT, $r=.685$ for thrombin time, $r=.107$ for Heptest^R, $r=.246$ for anti Xa and $r=.148$ for anti IIa). The correlation obtained for residual activity after CY

216 neutralization and clot score (r=.185 for APTT, r=.001 for thrombin time, r=.433 for Heptest^R, r=.473 for anti Xa and r=.153 for anti IIa).

2. Subcutaneous Studies

Clot scores obtained in the rabbit stasis thrombosis after the administration of heparin (1 mg/kg s.c.) or CY 216 (1 mg/kg s.c.) and subsequent antagonism by protamine sulfate (1 mg/kg i.v.) are shown in Figure 26. Heparin (1 mg/kg s.c.) and CY 216 (1 mg/kg i.v.) exhibited significant ($p<.05$), (as determined by a two way ANOVA) inhibition of thrombus formation as compared to the control group. Protamine sulfate (1 mg/kg i.v.) completely antagonized the antithrombotic effects of heparin (1 mg/kg s.c.). CY 216 exhibited a 30% inhibition of thrombosis as compared to control, however, this was not significant. Protamine sulfate did not antagonize the antithrombotic effects of CY 216 (1 mg/kg s.c.).

The coagulation parameters of rabbits administered subcutaneous heparin or CY 216 and protamine sulfate are shown in Table 9. Assay dependant differences in the neutralization of heparin and CY 216 were observed. The APTT was not significantly ($p>.50$), (as determined by a two way ANOVA) prolonged after heparin or CY 216 administration. The thrombin time for heparin (1 mg/kg s.c.) was prolonged to a greater extent than after the administration of CY 216. After equigravimetric protamine administration, the thrombin time activity of CY 216 was completely neutralized while this activity of heparin was substantially neutralized however not to baseline levels. The Heptest^R was significantly ($p<.05$) prolonged by heparin (1 mg/kg s.c.) and by CY 216 (1 mg/kg s.c.). Protamine completely neutralized the Heptest^R activity

of heparin, however not all of Heptest^{R} activity of CY 216 was neutralized.

Heparin (1 mg/kg s.c.) and CY 216 (1 mg/kg s.c.) significantly ($p<.05$) inhibited amidolytic factor Xa activity as shown in Figure 27. This activity was not neutralized by protamine (1 mg/kg i.v.) to control levels.

Heparin (1 mg/kg s.c). and CY 216 (1 mg/kg s.c.) exhibited a significant ($p<.05$), (as determined by a two way ANOVA), inhibition of the amidolytic activity of factor IIa as shown in Figure 28. Protamine neutralized this activity of heparin to a greater degree than CY 216.

The numerical values for the data plotted in Figures 18-27 are shown in Appendix XXIVg - XXIVj.

3. Rat Tail Bleeding Model

a. Intravenous Studies

The effects of saline, protamine (1 mg/kg i.v.), heparin (2 mg/kg i.v.) and CY 216 (2 mg/kg i.v.) are shown in Figure 29. Heparin (2 mg/kg i.v.) significantly ($p <.05$), (as determined by a one way ANOVA), prolonged the bleeding time while CY 216 or protamine had lesser effects on the bleeding time.

The antagonism of the bleeding effects of intravenously administered heparin and CY 216 by equigravimetric doses of protamine are shown in Figure 30. Heparin (2 mg/kg i.v.) significantly ($p<.05$), (as determined by a one way ANOVA) prolonged the bleeding time in this model, however, the bleeding time returned to control values after the administration of protamine (2 mg/kg i.v.). CY 216 did not significantly prolong the bleeding time in rats as compared to the control

group and subsequent protamine administration had no further effect on the bleeding time after subcutaneous administration of CY 216.

b. Subcutaneous Studies

The antagonism of subcutaneously administered heparin and CY 216 in a rat tail bleeding model is shown in Figure 31. Heparin (2.5 mg/kg s.c.) or CY 216 at (2.5 mg/kg s.c.) did not significantly ($p>.50$), (as determined by a one way ANOVA), prolong the bleeding time which was determined 2 hours after drug administration. Administration of protamine did not further affect the bleeding time.

The numerical values for the data plotted in Figures 29-31 are shown in Appendix XXIVb.

4. Rabbit Ear Blood Loss Model

The antagonism of heparin or CY 216 induced blood loss was studied in a rabbit ear model. Heparin and CY 216 were administered by intravenous and subcutaneous routes of administration and blood samples were obtained by cardiac puncture. Plasma was analyzed using the APTT, thrombin time, Heptest^{R}, amidolytic anti Xa and amidolytic anti IIa methods.

a. Intravenous Studies

1. Protamine Sulfate

The effect of protamine sulfate in a rabbit ear model of blood loss is shown in Figure 32. As previously described in methods, the red blood cells resulting from 5 full thickness cuts in the medial portion of the rabbit ear were collected in 1 liter of saline for 10 minutes at 37^{o}C. The cells were counted using a Hycel^{R} cell counter and

blood loss was reported as RBCs x 10^9/liter. Protamine sulfate (2 and 3 mg/kg i.v.) did not increase blood loss as compared to control.

Figure 33. demonstrates the ex vivo anticoagulant effect of protamine sulfate (2 and 3 mg/kg i.v.) administered to rabbits as determined by the APTT. Protamine prolonged the APTT in a dose related manner, however this increase in clotting time was not significant ($p>.01$) as determined by a one way ANOVA.

The effects of protamine sulfate administration to rabbits as determined by the thrombin time, Heptest^R, amidolytic anti Xa and amidolytic anti IIa methods are shown in Table 10. Protamine (2 and 3 mg/kg i.v.) did not prolong the clotting times to any great extent or have any effect on the amidolytic anti Xa or amidolytic anti IIa assays.

2. Heparin

The effect of heparin (1 and 2 mg/kg i.v.) is shown in Figure 34. Heparin produced a dose related increase in blood loss which was significant ($p <.05$) as compared to control as determined by a one way ANOVA.

The effects of heparin administration to rabbits as determined by the APTT, thrombin time and Heptest^R are shown in Table 11. The high doses of heparin used in the rabbit ear blood loss studies significantly prolonged the clotting times to > 150 seconds for the APTT and thrombin time and > 300 seconds for the Heptest^R.

The ex vixo amidolytic anti Xa activity after heparin administration to rabbits is shown in Figure 35. Heparin produced a dose related inhibition of the amidolytic action of factor Xa. Heparin (2 mg/kg

i.v.) caused a significant ($p < .05$) inhibition of factor Xa as compared to control as determined by a one way ANOVA.

Similarly, heparin inhibited the amidolytic action of factor IIa in a dose related manner. Figure 36 shows the ex vivo anti IIa activity of heparin after intravenous administration to rabbits. Heparin (1 and 2 mg/kg i.v.) significantly ($p < .05$) inhibited factor IIa as compared to control as determined by a one way ANOVA.

Correlation coefficients were calculated to determine the relationship between circulating pharmacodynamic activity and heparin-induced blood loss. Poor correlations ($r = .690$ for anti Xa and $r = .564$ for anti IIa) were obtained for the laboratory tests.

3. CY 216

The effect of CY 216 (1, 2 and 3 mg/kg i.v.) in a rabbit ear model of blood loss is shown in Figure 37. CY 216 produced a dose related increase in blood loss. CY 216 (3 mg/kg i.v.) produced significant ($p < 0.05$) increase in blood loss as compared to control as determined by a one way ANOVA.

The effects of CY 216 administered to rabbits as determined by the APTT, thrombin time and Heptest^{R} are shown in Table 12. The high doses of heparin used in the rabbit ear blood loss studies significantly prolonged the clotting times to > 150 seconds for the APTT and thrombin time and > 300 seconds for the Heptest^{R}.

The ex vivo amidolytic anti Xa activity of CY 216 after administration to rabbits is shown in Figure 38. CY 216 increased the inhibition of the amidolytic factor Xa activity in a dose related manner. Significant ($p < .05$) inhibition of amidolytic factor Xa

activity was seen at the three doses studied (1, 2 and 3 mg/kg i.v.) as compared to control as determined by a one way ANOVA.

Similarly, CY 216 produced a dose dependant inhibition of the amidolytic action of factor IIa. Figure 39 shows the ex vivo amidolytic anti IIa activity of CY 216 after administration to rabbits. CY 216 (1, 2 and 3 mg/kg i.v.) significantly ($p < .05$) inhibited factor IIa as compared to control as determined by a one way ANOVA.

Correlation coefficients were calculated to determine the relationship between circulating ex vivo activity and the CY 216 induced-blood loss. Poor correlations were obtained ($r = .515$ for Heptest^R, $r = .102$ for anti Xa and $r = .186$ for anti IIa) for the various laboratory tests.

4. Protamine Antagonism of the Effects of Heparin and CY 216

a. Intravenous Studies

The protamine antagonism of heparin (2 mg/kg i.v.) and CY 216 (2 mg/kg i.v.) induced blood loss is shown in Figure 40. Heparin (2 mg/kg i.v.) produced a significant ($p < .05$), (as determined by a two way ANOVA), increase in blood loss as compared to control. The blood loss induced by CY 216 was completely antagonized by an equigravimetric amount of protamine, however a two fold gravimetric amount of protamine was needed to completely antagonize the blood loss induced by heparin.

Ex vivo coagulation parameters as determined by the APTT, thrombin time and Heptest^R obtained after heparin or CY 216 and protamine administration in rabbits are shown in Table 13. Although the clotting times were significantly prolonged by heparin (2 mg/kg i.v.) and CY 216 (2 mg/kg i.v.), protamine completely neutralized the

APTT activity at equigravimetric amounts of protamine and heparin or CY 216. The thrombin time activity was completely neutralized at a gravimetric heparin (or CY 216) to protamine ratio of 1:2. Although protamine (3 mg/kg i.v.) significantly neutralized the Heptest^{R} activity of heparin (2 mg/kg i.v.), CY 216 was not completely neutralized at this dose. Significant ($p<.05$), (as determined by a two way ANOVA), activity remained after protamine (3 mg/kg i.v.) administration to rabbits which received CY 216 (2 or 3 mg/kg i.v.) as compared to the control group.

The ex vivo amidolytic anti Xa activity after protamine neutralization of heparin or CY 216 administered to rabbits is shown in Figure 41. Both heparin and CY 216 significantly ($p<.05$) inhibited the amidolytic actions of factor Xa as determined by a two way ANOVA. Protamine (2 and 3 mg/kg i.v.) completely neutralized the anti Xa activity of heparin, however these doses of protamine did not completely neutralize the anti Xa activity of CY 216. Significant ($p<.05$) inhibition of factor Xa remained after protamine (2 and 3 mg/kg i.v.) administration. The ex vivo anti IIa activity after protamine neutralization of heparin or CY 216 in rabbits is shown in Figure 42. Both heparin and CY 216 caused a significant increase in the inhibition of factor IIa. Protamine (2 and 3 mg/kg i.v.) completely neutralized this activity.

The correlation coefficients obtained for residual activity after heparin neutralization and blood loss are as follows: $r=.421$ for APTT, $r=.765$ for thrombin time, $r=.391$ for Heptest^{R}, $r=0$ for anti Xa and $r=0$ for anti IIa. The correlation obtained for residual activity after CY

216 neutralization and blood loss are as follows: r=.576 for APTT, r=.812 for thrombin time, r=.460 for anti Xa and r= 0 for anti IIa.

b. Subcutaneous Studies

Heparin and CY 216 (3 mg/kg s.c.) did not increase the blood loss which was determined 2 hours after the administration of these agents. Protamine (3 mg/kg i.v.) administration to rabbits did not have any additional effects on blood loss as shown in Figure 43.

The neutralization of heparin (3 mg/kg s.c.) and CY 216 (3 mg/kg s.c.) by equigravimetric protamine as determined by APTT, thrombin time and Heptest^{R} is shown in Table 14. Although the clotting times were elevated by heparin (3 mg/kg s.c.), protamine completely neutralized the APTT and thrombin time activity at a gravimetric protamine to heparin ratio of 1:1. After administration of heparin (3 mg/kg s.c.) the Heptest^{R} clotting time was significantly prolonged ($p<.05$), (as determined by a two way ANOVA). Protamine (3 mg/kg i.v.) neutralized the Heptest^{R} activity to almost control levels. CY 216 (3 mg/kg s.c.) did not prolong the APTT. The thrombin time activity of CY 216 (3 mg/kg s.c.) was completely neutralized at equigravimetric protamine dose and CY 216 (3 mg/kg s.c.) significantly ($p<.05$) prolonged the Heptest^{R}, however, this activity was not neutralized by protamine (3 mg/kg i.v.).

The ex vivo amidolytic anti Xa activity after protamine neutralization of heparin or CY 216 in rabbits is shown in Figure 44. Both heparin (3 mg/kg s.c.) and CY 216 (3 mg/kg s.c.) exhibited significant ($p<.05$) inhibition of amidolytic factor Xa activity as determined by a two way ANOVA. Heparin (3 mg/kg s.c.) produced an inhibition of the

amidolytic action of factor Xa which was completely neutralized by protamine (3 mg/kg i.v.) while, this activity of CY 216 (3 mg/kg s.c.) was not completely neutralized.

The ex vivo amidolytic anti IIa activity after protamine neutralization of heparin or CY 216 in rabbits is shown in Figure 45. Both heparin (3 mg/kg s.c.) and CY 216 (3 mg/kg s.c.) exhibited significant ($p<.05$) inhibition of amidolytic factor IIa activity as determined by a two way ANOVA. The amidolytic anti IIa activity of heparin (3 mg/kg s.c.) and CY 216 (3 mg/kg s.c.) was completely neutralized by protamine (3 mg/kg i.v.).

The numerical values for the data plotted in Figures 32-45 are shown in Appendix XXIV.

5. Protamine Administration to Primates

The time course of the anticoagulant effects of protamine (2.1 mg/kg i.v.) administration to primates is shown in Figure 46. This dose of protamine had minimal effects on clotting as determined by the APTT, thrombin time and Heptest^{R} assays. A slight decrease in thrombin time activity was seen immediately after protamine administration, however this activity returned to control values by 30 minutes.

The time course of the antiprotease effects protamine (2.1 mg/kg i.v.) in primates as determined by amidolytic anti Xa and anti IIa assays is shown in Figure 47. Protamine (2.1 mg/kg i.v.) had no effect on amidolytic anti Xa activity, however, it produced a significant increase ($p<.05$),(as determined by a two way ANOVA), in the inhibition of factor IIa as compared to control values. As shown earlier in Figures 46 and 47, the clotting and amidolytic methods were not very

sensitive to the effects of protamine sulfate. In order to determine the time course of protamine sulfate activity in primates, ex vivo plasma samples obtained from the primates administered protamine (2.1 mg/kg i.v.) were supplemented with heparin (25 μg/ml to obtain a final heparin concentration of 2.5 μg/ml. The degree of neutralization of the Heptest^{R} activity for the heparin (2.5 μg/ml) by protamine in the exvivo plasma sample was an indirect measure of the circulating levels of protamine activity. The results from this study are shown in Figure 48. At 5 minutes after supplementation a measurable amount decrease in heparin activity suggesting protamine activity. Statistical analysis by a two way ANOVA revealed no significant differences in Heptest^{R} activity at the dosages studied, however this data suggests that the $t_{1/2}$ of protamine is less than 30 minutes.

6. Time Course of The Protamine Neutralization of Heparin and CY 216 in Primates

Protamine neutralization of the pharmacodynamic time course of intravenously and subcutaneously administered heparin and CY 216 was studied in the primate (Macaca mulatta). Activity of heparin and CY 216 was determined using APTT, thrombin time, Heptest^{R}, amidolytic anti Xa and amidolytic anti IIa methods. Pharmacodynamic time course parameters were calculated from concentrations obtained from Heptest^{R} and amidolytic anti Xa activity data which was extrapolated from calibration curves of heparin or CY 216 supplemented to NMP.

a. Intravenous Administration

The time course of APTT activity of heparin (0.7 mg/kg i.v.) prior to and after neutralization by protamine sulfate (0.7, 1.4 and

2.1 mg/kg i.v.) is shown in Figure 49. After administration of heparin, the clotting time was > 150 seconds and remained elevated above baseline until 3 hours after injection. Complete neutralization of APTT activity was seen immediately after protamine sulfate (1.4 mg/kg i.v. and 2.1 mg/kg i.v.) administration. However, significant ($p<.05$) APTT activity remained 5 minutes after protamine (0.7 mg/kg i.v.) administration as determined by a two way ANOVA. Partial neutralization of the APTT activity of heparin by protamine sulfate (0.7 mg/kg i.v.) was observed.

The time course of thrombin time activity of heparin (0.7 mg/kg i.v.) prior to and after neutralization by protamine sulfate (0.7, 1.4 and 2.1 mg/kg i.v.) is shown in Figure 50. After administration of heparin, the clotting time was > 150 seconds and returned to baseline at 6 hours. Complete neutralization of thrombin time activity was seen for protamine (1.4 and 2.1 mg/kg i.v.) at 60 minutes. Wide variation in the neutralization by protamine (0.7 mg/kg i.v.) was seen during the first hour after protamine administration.

The time course of Heptest^R activity of heparin (0.7 mg/kg i.v.) prior to and after neutralization by protamine sulfate (0.7, 1.4 and 2.1 mg/kg i.v.) is shown in Figure 51. After administration of heparin (0.7 mg/kg i.v.), the clotting time was > 300 seconds and returned to baseline values by 6 hours. Protamine sulfate substantially neutralized the Heptest^R activity of heparin (0.7 mg/kg i.v.). Complete neutralization of Heptest^R activity by protamine (2.1 mg/kg i.v.) was seen at 1 hour while complete neutralization of Heptest^R activity by protamine (1.4 and 0.7 mg/kg i.v.) occurred by 3 hours.

The time course of the amidolytic anti Xa activity of heparin (0.7 mg/kg i.v.) prior to and after neutralization by protamine sulfate (0.7, 1.4 and 2.1 mg/kg i.v.) is shown in Figure 52. After administration of heparin (0.7 mg/kg i.v.), the % inhibition of amidolytic factor Xa activity was 92% which returned to baseline activity by 6 hours. Dose dependant protamine neutralization of this anti Xa activity was seen.

The time course of the amidolytic anti IIa activity of heparin (0.7 mg/kg i.v.) prior to and after neutralization by protamine sulfate (0.7, 1.4 and 2.1 mg/kg i.v.) is shown in Figure 53. After administration of heparin (0.7 mg/kg i.v.) the % inhibition of factor IIa was 91 % which did not completely return to baseline until 6 hours. At the 3 hour time period, complete neutralization of the amidolytic anti IIa activity of heparin was seen with all doses of protamine sulfate administered. The time course of APTT activity of CY 216 (0.7 mg/kg i.v.) prior to and after neutralization by protamine sulfate (0.7, 1.4 and 2.1 mg/kg i.v.) is shown in Figure 54. CY 216 (0.7 mg/kg i.v. and 0.35 mg/kg i.v.) prolonged the clotting time to a lesser degree than heparin at the same dose. Complete neutralization of the APTT activity of CY 216 was seen after the administration of protamine (0.7, 1.4 and 2.1 mg/kg i.v.).

The time course of thrombin time activity of CY 216 (0.7 mg/kg i.v.) prior to and after neutralization by protamine sulfate (0.7, 1.4 and 2.1 mg/kg i.v.) as shown in Figure 55. CY 216 (0.7 mg/kg i.v.) prolonged the clotting time to a lesser degree than heparin at the same dose. While neutralization of the thrombin time activity of CY 216 was

seen after the administration of protamine (1.4 mg/kg i.v.). Complete neutralization of thrombin time activity by protamine (0.7 mg/kg i.v.) was seen at 1 hour.

The time course of Heptest^{R} activity of CY 216 (0.7 mg/kg i.v.) prior to and after neutralization by protamine sulfate (0.7, 1.4 and 2.1 mg/kg i.v.) is shown in Figure 56. CY 216 (0.7 mg/kg i.v.) prolonged the Heptest^{R} to > 300 seconds. CY 216 (0.7 mg/kg i.v.) did not prolong the Heptest^{R} to the same extent as did the same concentrations of heparin. Protamine sulfate (0.7, 1.4 and 2.1 mg/kg i.v.) did not neutralize the Heptest^{R} activity of CY 216 to any significant extent.

The time course of the amidolytic anti Xa activity of CY 216 (0.7 mg/kg i.v.) prior to and after neutralization by protamine sulfate (0.7, 1.4 and 2.1 mg/kg i.v.) is shown in Figure 57. Inhibition of the amidolytic actions of factor Xa by CY 216 (0.7 mg/kg i.v.) was seen for over 6 hours. Dose dependant neutralization of this activity of CY 216 was seen by protamine (0.7 mg/kg i.v.). Statistically significant ($p<.05$) differences in neutralization were seen after protamine (0.7 mg/kg i.v.) and protamine (2.1 mg/kg i.v.) as determined by a two way ANOVA. Complete neutralization of the factor Xa inhibition was not seen at these doses of protamine studied.

The time course of amidolytic anti IIa activity of CY 216 (0.7 mg/kg i.v.) prior to and after neutralization by protamine sulfate (0.7, 1.4 and 2.1 mg/kg i.v.) is shown in Figure 58. Inhibition of the amidolytic action of factor IIa by CY 216 (0.7 mg/kg i.v.) was seen at 3 hours. Dose dependant neutralization of the anti II activity of CY

216 (0.7 mg/kg i.v) was seen, with complete neutralization of anti IIa activity at 3 hours. With protamine (0.7 mg/kg i.v.), a slight rebound of anti IIa activity which returned to baseline after 3 hours.

b. Subcutaneous Studies

The time course of Heptest^R activity after administration of heparin (0.7 mg/kg s.c.) prior to and after neutralization by protamine sulfate (0.7 mg/kg i.v.) as shown in Figure 59. Protamine was administered at 4 hours, at a time when the heparin activity was strong. Complete neutralization of the Heptest^R activity of heparin was seen at this time.

As shown in Figure 60, CY 216 was not readily neutralized by protamine sulfate in the Heptest^R assay. Even two fold gravimetric amount of protamine sulfate did not neutralize this activity of subcutaneously administered CY 216. At 9 hours, after the administration of protamine the Heptest^R clotting time was prolonged to almost two times the baseline levels.

The time course of the neutralization of heparin (0.7 mg/kg s.c.) as shown in Figure 61. Low levels of amidolytic anti Xa inhibition were noted after the administration of heparin (0.7 mg/kg s.c.) and no changes in the amidolytic anti Xa activity after protamine administration of this activity was seen. Heparin contains less low molecular weight components than CY 216 and therefore exhibits less anti Xa activity after subcutaneous administration.

The time course of the neutralization of amidolytic anti Xa activity of CY 216 (0.7 mg/kg s.c.) is shown in Figure 62. After protamine administration, at 4 hours, CY 216 inhibited the amidolytic

anti Xa activity approximately 50 %. As time continued, this activity of the group of primates which received protamine closely followed the time course of the group which received CY 216 (0.7 mg/kg s.c.). This data suggests that protamine initially neutralized the anti Xa activity of CY 216 but failed to neutralize the activity of CY 216 which was absorbed at a later time period.

c. Pharmacodynamic Time Course Parameters

Pharmacodynamic time course parameters were calculated using a noncompartmental model as previously described in methods. Concentrations (μg/ml) were obtained after extrapolation from calibration curves of anti Xa or Heptest^{R} activity of heparin and CY 216 after supplementation to NMP.

The numerical values for the data plotted in Figures 49-62 are shown in Appendix XXV.

1. Anti Xa

a. Intravenous Studies

The pharmacodynamic time course parameters for heparin (0.35 mg/kg i.v). and CY 216 (0.35 mg/kg i.v). as calculated from amidolytic anti Xa activity data are shown in Table 15. A comparison of these parameters reveal that the AUC: CY 216 > heparin , MRT: CY 216 > heparin, Clp: heparin > CY 216 and Vd: heparin > CY 216. The AUC, MRT and Clp of CY 216 were significantly ($p<.05$) than the similar parameters for heparin as determined by an unpaired student's t test.

Table 16 shows the pharmacodynamic time course parameters for heparin (0.7 mg/kg i.v). and CY 216 (0.7 mg/kg i.v.) as calculated from amidolytic anti Xa activity data. A comparison of these parameters

reveal that the AUC: heparin > CY 216, MRT: CY 216 > heparin, Clp: CY 216 > heparin, Vd: CY 216 > heparin. However there was no significant ($p>.10$) difference between the AUC, MRT, Clp and Vd values of heparin (0.7 mg/kg i.v). and CY 216 (0.7 mg/kg i.v.) as determined by an unpaired student's t test.

When protamine sulfate was administered in equigravimetric doses to heparin (0.7 mg/kg i.v.) or CY 216 (0.7 mg/kg i.v.), the pharmacodynamic time course parameters obtained are shown in Table 17. A comparison of these parameters revealed that the AUC: CY 216 > heparin, MRT: CY 216 > heparin, PNI: heparin > CY 216. However, AUC and PNI values after equigravimetric neutralization of heparin or CY 216 by protamine (0.7 mg/kg i.v.) were significantly different as determined by an unpaired student's t test.

When protamine sulfate (1.4 mg/kg i.v.) was administered in a two fold gravimetric dose to heparin (0.7 mg/kg i.v.) or CY 216 (0.7 mg/kg i.v.), the pharmacodynamic time course parameters obtained are shown in Table 18. A comparison of these parameters revealed that following: AUC: CY 216 > heparin, MRT: CY 216 > heparin and PNI: heparin > CY 216. The AUC of CY 216 (0.7 mg/kg i.v.) and protamine (1.4 mg/kg i.v.) was significantly greater ($p <.05$) than the AUC of heparin (0.7 mg/kg i.v.) and protamine (1.4 mg/kg i.v.) as determined by an unpaired student's t test. The PNI was also significantly ($p<.05$) greater for heparin (0.7 mg/kg i.v) and protamine (1.4 mg/kg i.v.) than CY 216 (0.7 mg/kg i.v.) and protamine (1.4 mg/kg i.v.).

When protamine sulfate (2.1 mg/kg i.v) was administered in a three fold gravimetric dose of heparin (0.7 mg/kg i.v.) and CY 216 (0.7

mg/kg i.v.), the primate pharmacodynamic time course parameters obtained are shown in Table 19. A comparison of these parameters revealed that following: AUC: CY 216 > heparin, MRT: heparin > CY 216, and PNI: heparin > CY 216. The AUC of CY 216 (0.7 mg/kg i.v.) and protamine (2.1 mg/kg i.v.) was significantly greater ($p<.05$) than the AUC of heparin (0.7 mg/kg i.v.) and protamine (2.1 mg/kg i.v.) as determined by an unpaired student's t test. The PNI was significantly greater ($p<.05$) for heparin (0.7 mg/kg i.v.) and protamine (2.1 mg/kg i.v.) than for CY 216 (0.7 mg/kg i.v) and protamine (2.1 mg/kg i.v.).

In summary, while studying the neutralization intravenously administered heparin and CY 216, the AUC of CY 216 was generally greater than the AUC of heparin after neutralization by protamine sulfate. In most cases the MRT of CY 216 was longer than that of heparin, in most cases. After protamine administration, the PNI of heparin was greater than the PNI of CY 216 which indicated that heparin was more neutralizable than CY 216 as determined by the anti Xa method.

b. Subcutaneous Studies

The pharmacodynamic time course parameters for heparin (0.7 mg/kg s.c.) and CY 216 (0.7 mg/kg s.c.) as calculated from amidolytic anti Xa activity data are shown in Table 20. A comparison of these parameters revealed that the AUC: CY 216 > heparin, MRT: CY 216 > heparin, Clp: heparin > CY 216, and Vd: heparin > CY 216. The AUC, MRT, Clp, Vd of CY 216 (0.7 mg/kg s.c.) were significantly different ($p<.05$) than similar parameters of heparin (0.7 mg/kg s.c.) as determined by an unpaired student's t test.

When protamine was administered in equigravimetric doses to primates which received heparin (0.7 mg/kg s.c.) or CY 216 (0.7 mg/kg s.c.), the following pharmacodynamic time course parameters which were obtained are shown in Table 21. A comparison of these parameters reveal that the AUC: CY 216 > heparin, MRT: heparin > CY 216, PNI: heparin > CY 216. The AUC of CY 216 was significantly ($p<.05$) greater than the AUC of heparin as determined by an unpaired student's t test. No significant differences were seen between the other pharmacodynamic time course parameters.

When protamine was administered in two fold gravimetric doses to primates given CY 216, the pharmacodynamic time course parameters which were obtained are shown in Table 22. There was no difference in the time course parameters of CY 216 (0.7 mg/kg s.c.) and protamine (0.7 or 1.4 mg/kg i.v.).

2. Heptest^R

a. Intravenous Studies

The pharmacodynamic time course parameters for heparin (0.35 mg/kg i.v.) and CY 216 (0.35 mg/kg i.v.) as calculated from Heptest^R activity data are shown in Table 23. A comparison of these parameters reveal that the AUC: CY 216 > heparin, MRT: CY 216 > heparin, Clp: heparin > CY 216, Vd: heparin > CY 216. Significant differences were seen for AUC, MRT ($p<.05$) and Clp ($p<.05$) of heparin and CY 216 as determined by an unpaired student's t test.

The pharmacodynamic time course parameters for heparin (0.7 mg/kg i.v.) and CY 216 (0.7 mg/kg i.v.) as calculated by the Heptest^R are shown in Table 24. A comparison of these parameters reveal that the

AUC: CY 216 > heparin, MRT: heparin > CY 216, Clp: heparin > CY 216, Vd: heparin > CY 216. Significant ($p<.05$) differences were seen between the AUC and MRT values of heparin (0.7 mg/kg i.v.) and CY 216 (0.7 mg/kg i.v.) as determined by an unpaired student's t test.

When protamine sulfate was administered in equigravimetric doses to heparin (0.7 mg/kg i.v.) and CY 216 (0.7 mg/kg i.v.), the pharmacodynamic time course parameters which were obtained are shown in Table 25. A comparison of these parameters reveal that the AUC: CY 216 >heparin, MRT: CY 216 > heparin, PNI: heparin = CY 216. However, the AUC was the only parameter of CY 216 which was significantly ($p<.05$) different from heparin as determined by an unpaired student's t test.

When protamine sulfate was administered in twice gravimetric dosages to heparin (0.7 mg/kg i.v.) and CY 216 (0.7 mg/kg i.v.) the following primate pharmacodynamic time course parameters were obtained as shown in Table 26. A comparison of these parameters reveal that the AUC: CY 216 >heparin, MRT: heparin > CY 216, PNI: heparin > CY 216. the AUC, MRT and PNI values of CY 216 (0.7 mg/kg i.v.) after the administration of two fold gravimetric amounts of protamine were significantly ($p<.05$) different as determined by an unpaired student's t test.

When protamine sulfate was administered in three times gravimetric dosages to heparin (0.7 mg/kg i.v.) and CY 216 (0.7 mg/kg i.v.) the following primate pharmacodynamic time course parameters were obtained as shown in Table 27. A comparison of these parameters reveal that the results are AUC: CY 216 >heparin, MRT: CY 216 > heparin, PNI: heparin > CY 216. After protamine (2.1 mg/kg i.v.) neutralization of

heparin (0.7 mg/kg i.v.) and CY 216 (0.7 mg/kg i.v.) the AUC and PNI of CY 216 was significantly ($p<.05$) different than for heparin.

b. Subcutaneous Studies

The pharmacodynamic time course parameters for heparin (0.7 mg/kg s.c.) and CY 216 (0.7 mg/kg s.c.) as calculated from Heptest^R activity data are shown in Table 28. A comparison of these parameters reveal that the AUC: CY 216 > heparin, MRT:heparin > CY 216, Clp: heparin > CY 216, Vd: heparin = CY 216. The AUC was the only parameter which was significantly ($p<.05$) different between the heparin and CY 216 groups. Bioavailability after subcutaneous administration of heparin and CY 216 as determined by the amidolytic anti Xa assay was calculated. CY 216 (0.7 mg/kg s.c.) exhibited 80% greater bioavailability than heparin (0.7 mg/kg s.c.)

The pharmacodynamic time course parameters for heparin (0.7 mg/kg s.c.) with protamine (0.7 mg/kg i.v.) and CY 216 (0.7 mg/kg s.c.) with protamine (0.7 mg/kg i.v.) as calculated from the Heptest^R method are shown in Table 29. A comparison of these parameters reveal that the results are AUC: CY 216 > heparin, MRT: CY 216 > heparin and PNI: heparin > CY 216. Significant ($p<.05$) differences between the AUC, MRT and PNI of heparin and CY 216 were observed as determined by an unpaired student t test.

The primate pharmacodynamic time course parameters obtained when protamine (1.4 mg/kg i.v.) was administered to primates given CY 216 (0.7 mg/kg s.c.) are shown in Table 30. A comparison of these parameters reveal that the AUC < for CY 216 (0.7 mg/kg s.c.) and protamine (0.7 mg/kg i.v.) than for CY 216 (0.7 mg/kg s.c.) and protamine (1.4

mg/kg i.v.) than when equigravimetric protamine was given to neutralize CY 216 (0.7 mg/kg s.c.). The MRT and PNI were slightly increased as compared to CY 216 (0.7 mg/kg s.c.) and protamine (0.7 mg/kg i.v.).

7. Comparative % Neutralization of Heparin and CY 216 in the Animal Models

Per cent neutralization values of the ex vivo activities obtained in the animal models were calculated as previously described in methods. These calculations provided a means of comparison of the degree of neutralization observed in the animal models at the various doses studied. Per cent neutralization values which were calculated at gravimetric protamine to heparin ratio of 1:1, 2:1 and 3:1 were compared for the intravenous studies are shown in Table 31 (anti Xa) and Table 32 (anti IIa). Per cent neutralization values for the subcutaneous studies are shown in Table 33 (anti Xa) and Table 34 (anti IIa).

Lower % neutralization values were observed for CY 216 as compared to heparin as determined by the amidolytic anti Xa and anti IIa assays. In rabbits, the amidolytic anti IIa activity was completely neutralized at a gravimetric protamine to heparin ratio of 2:1, while the amidolytic anti Xa activity was not completely neutralized. After subcutaneous administration of heparin a decreased amount of neutralization was observed for most cases as compared to intravenous administration.

CHAPTER V

DISCUSSION

Heparin has been used effectively as an antithrombotic drug for over 50 years, however its multicomponent structure makes the complete elucidation of its mechanism of action difficult. Commercial heparin is heterogenous with respect to internal structure (Casu 1984), molecular size (Andersson 1979), degree of sulfation (Hurst et al. 1979) and anticoagulant activity (Hurst et al. 1981). The molecular heterogeneity within heparin preparations contributes to the complexity of the interaction of this drug with components of the hemostatic system. Heparin interacts with platelets (Salzman 1980), plasma proteins (Rosenberg et al. 1985) and the endothelium (Barzu 1985) and depending upon the dose and route of administration produces anticoagulant, antithrombotic or antihemostatic effects.

The anticoagulant effects of heparin are the result of heparin forming a complex with AT III and inhibiting activated coagulation factors via this plasma cofactor. This activity of heparin has been used for many years to monitor heparin therapy through the use of global tests such as the APTT and thrombin time. This effect of heparin is differentially related to molecular weight. Anticoagulant activity decreases with decreasing molecular weight while both low and high molecular weight heparins can exert antithrombotic activity.

The antithrombotic effects of heparin are related to the ability of heparin to inhibit thrombus formation in vivo. Components of

heparin with high affinity to antithrombin III can exert antithrombotic effects and low affinity heparin can potentiate these effects (Barrowcliffe 1984).

Antihemostatic effects of heparin occurring after high dose administration may result from the inhibition of coagulation enzymes and platelets and cause bleeding. The antihemostatic effects of heparin are influenced by a number of variables including dose and patient characteristics.

Fractionation and depolymerization of heparin by a variety of techniques (Casu 1985) results in the production of heparin derivatives with a decreased molecular weight, an increased ability to inhibit factor Xa and a decreased ability to inhibit thrombin. These compounds were developed in order that the heparin derivatives would retain antithrombotic activity and exhibit reduced hemorrhagic potential. Low molecular weight heparins are currently being developed clinically for the prophylaxis and therapy of thrombosis (Samama 1989) and these therapeutic agents exhibit certain advantages as compared to heparin. At equivalent doses, they cause a decreased amount of blood loss as compared to standard heparin in man (Kakaar et al. 1985; Schmitz-Huebner et al. 1984;Turpie et al. 1986) and they only require once daily subcutaneous injections for the prophylaxis of thrombosis as compared to 2-3 times daily for heparin.

Low molecular weight heparins exhibit decreased anticoagulant activity per mg as determined by clotting tests such as the APTT and thrombin time. These agents contain a decreased proportion of molecules with a molecular weight > 5600 daltons which is the minimum

molecular weight required to inhibit thrombin. The degree of prolongation of the APTT by heparins is related to the % of compounds of low molecular weight heparin with a molecular weight less than 5600 daltons.

A large proportion of the molecules contained within low molecular weight heparins inhibit factor Xa. This factor occupies a central position in the coagulation cascade where the extrinsic and intrinsic pathways meet to form the common pathway of coagulation. Factors Xa, V, phospholipid and Ca^{+2} form the prothrombinase complex which is involved in the formation of thrombin. Low molecular weight heparins exhibit antithrombotic actions through the inhibition of thrombin generation, while unfractionated heparins, inhibit thrombin generation in addition to thrombin activity.

Low molecular weight heparin produce less antihemostatic effects than equivalent dosages of heparin (Kakaar et al. 1986). This decrease in blood loss may be related to a decreased proportion of components with high molecular weight and high charge density contained within the low molecular weight heparin. These components are reported to inhibit thrombin and collagen-induced platelet aggregation (Cade 1984).

Although the use of low molecular weight heparin is associated with less bleeding than heparin, under certain circumstances it may be necessary to neutralize the blood loss induced by low molecular weight heparin. These instances include: overdose or reversal of anticoagulation following procedures involving extracorporeal circulation such as cardiopulmonary bypass and hemodialysis for renal failure. Although protamine is administered specifically to neutralize the bleeding

induced by heparin, protamine also influences the anticoagulant, antiprotease and antithrombotic activities of the heparins.

Protamine has been used for many years to neutralize the bleeding induced by heparins, and protamine antagonism of heparin has been associated with a variety of adverse effects. In addition, assays with increased sensitivity toward low molecular weight heparins have been developed which can detect activities of heparins which are not completely neutralizable by protamine. Protamine has been shown to exhibit a differential degree of neutralization in these assays (Racanelli et al. 1985). Although in vitro assays are useful to predict the hemorrhagic potential of heparins, the relevance of ex vivo laboratory parameters of experimental animals and patients receiving heparin and protamine requires further investigation.

Preliminary in vitro studies (Racanelli et al. 1985) have demonstrated that low molecular weight heparins exhibit characteristic protamine neutralization profiles which differ significantly from the neutralization profiles of heparin. In addition, the protamine neutralization profile of heparin and low molecular weight heparins are assay dependant. After supplementation of heparin and CY 216 in vitro to NHP, the clotting and anti IIa activities of the heparins are more readily neutralized by protamine than the anti Xa activities of the heparins.

A variety of approaches utilizing in vitro assays and in vivo models of thrombosis and bleeding were used to study the antagonism of heparin and CY 216 by protamine. The in vitro assays were targeted to different sites in the coagulation cascade in order to provide a

sensitive means of determining the activity of the heparins. Animal models of thrombosis with differing activation mechanisms, and animal models of bleeding were utilized to address the *in vivo* antagonism of heparins. This integrative approach was used to determine whether the *in vivo* neutralization profiles of heparin and CY 216 were similar and whether partial neutralization of low molecular weight heparins which was observed *in vitro* contributes to hemorrhagic or antithrombotic effects. The current study also determined whether protamine administration altered the pharmacodynamic time course parameters of heparin and CY 216. An integrated approach to the study of the neutralization of heparin and CY 216 has provided information on the endogenous behavior of the heparins after antagonism by protamine which may influence the management of patients undergoing antithrombotic therapy.

A. The Assessment of the Potency of Heparins

When heparin was first introduced into therapeutic use, its *in vitro* anticoagulant action was equated with its *in vivo* antithrombotic effects. However, the mechanism of action of heparin is complex and an ideal assay for heparin has not yet been developed. "Laboratory evaluation of the efficacy of heparin therapy is an inherently complex problem, as it entails the assessment of the effects of a variable and heterogenous mixture of substances on a complex biological system whose actions and interactions are incompletely understood" (Gawoski 1987).

The assays in routine use for determining heparin potency are either based upon the overall anticoagulant activity of heparin or on specific properties of heparin such as the inactivation of thrombin or

factor Xa (Dawes et al. 1986.). The APTT remains the most commonly used assay for monitoring heparin effects, however it is less sensitive to the effects of low molecular weight heparin since it measures thrombin inhibition rather than the inhibition of the other activated coagulation factors such as factor Xa. An amplification mechanism within the coagulation cascade exists such that a ten fold fold molar increase occurs at each step in the cascade and only a low level of thrombin is required to generate clot formation (McFarlane 1964). Nearly a complete inhibition of factor Xa which is generated during clotting is required to inhibit thrombin generation, therefore the APTT may be more sensitive to components of heparin which inhibit thrombin rather than factor Xa (Ofosu et al. 1985). Plasma based global assays such as APTT which use physiologic concentrations of activators and inhibitors may be more sensitive to agents which inhibit thrombin as opposed to the other coagulation factors.

Recently, more specific tests have been developed where activated enzymes are supplemented to heparinized plasma in order to identify the various sites of heparin action. The Heptest^{R} which was developed by Yin (1983), represents a modified version of the APTT which is more sensitive to low molecular weight heparins since exogenous factor Xa is used. This clot based anti Xa/IIa method, may have an advantage over amidolytic assays in that it utilizes undiluted plasma and a clotting endpoint. Amidolytic assays have been useful in the study the neutralization of heparins and have added a degree of specificity in the determination of coagulation enzyme inhibition. Isolated enzyme assay systems, however, may not reflect the physiologic or true function of

the coagulation enzymes and inhibitors because they use diluted plasma and synthetic substances.

It was necessary, therefore in these studies to determine heparin concentration indirectly by using assays which measure heparin activity. The in vitro clotting assays were used in these studies to determine the collective action of the heparins on the coagulation system. Although these assays are similar in their method of end point detection (clot formation), they differ in the sensitivity to the various sites within the coagulation cascade to which the heparins are targeted. The APTT measures the intrinsic system, the thrombin time assay measures the conversion of fibrinogen to fibrin and the Heptest^{R} measures the collective inhibitory effects at the level of factor Xa and factor IIa. The amidolytic anti Xa and amidolytic anti IIa assays, on the other hand, measure the specific ability of the heparins to inhibit the amidolytic activity of factor Xa or factor IIa.

B. Selection of Doses Used in the Studies

Gravimetric amounts of heparin, CY 216 and protamine were compared in the in vitro assays and in the animal models. Administration of the drugs was carried out in gravimetric amounts for several reasons. First, administration of polycomponent drugs on a unitage basis introduces a bias toward the specific activity on which the unit is based. Studies by Fareed et al. (1988) have shown that potency adjustment of low molecular weight heparins based upon amidolytic anti Xa activity does not minimize the variations of the heparins observed in other in vitro assays or in animal models of thrombosis and bleeding. Second, it is not appropriate to administer molar amounts of the

heparins. Using the mean molecular weight to calculate approximate molar amounts of an equigravimetric amount of CY 216 and heparin, it was found that CY 216 contains approximately twice the number of molecules as heparin. However, since heparin and CY 216 are distributed over a fairly wide molecular weight range, this calculation was not very accurate. Furthermore, in addition to containing a heterogenous mixture of components with varying chain lengths, heparins also exhibit internal structural microheterogenity since all heparin components do not contain the identical chemical sequences (Casu 1985). Differences are noted in the degree of sulfation (Hurst 1979) and affinity toward AT III (Casu 1985) of the individual heparin molecules.

A series of experiments were performed in order to investigate the feasibility of using molar amounts in the study of the antagonism of heparin and CY 216. A laser-induced thrombosis model was used for these studies. One mg amounts of heparin and CY 216 were converted to apparent molar amounts, as shown in Appendix XVI, and this molar quantity of protamine was administered to rats. No additional information about the comparative protamine neutralization of heparin and CY 216 was obtained when the drugs were administered in approximate equimolar amounts. In fact, comparisons of heparins based on apparent molar amounts led to completely opposite conclusions regarding the degree of protamine neutralization of heparin and CY 216 as when gravimetric doses of these drugs are administered. When apparent equivalent molar amounts were used the antithrombotic activity of heparin appeared to be less neutralizeable than the antithrombotic activity of CY 216. As our studies have demonstrated using the rabbit

stasis thrombosis model and as others have reported (Dinness 1986), the antithrombotic effects of heparin were neutralized to a greater degree than the antithrombotic effects of low molecular weight heparins by protamine. In addition, the therapeutic use of heparin involves administration of U/mg amounts of heparin to gravimetric amounts of protamine.

The doses which were used in the animal experiments were selected in order that the plasma concentrations were in the approximate range of clinically used heparin and protamine. Appendix XVII shows the doses used in the animal models and the doses of heparin and protamine used for cardiopulmonary bypass at Foster G. McGaw Hospital. Higher doses (1-3 mg/kg) of heparin and CY 216 were required to induce blood loss in the animal models than were required to inhibit thrombosis (25-100 μg/kg). The doses used in the rat tail bleeding model and rabbit ear blood loss model were in the approximate range of doses which are used during cardiopulmonary bypass procedures. The gravimetric ratio of protamine:heparin of 1.4:1 which was used in the primate studies was similar to the ratio of protamine to heparin which is used clinically (1.3 mg of protamine to 1 mg of heparin).

C. Molecular Weight Determination

Gel permeation chromatography was used to determine the molecular weight profiles of heparin, CY 216 and protamine. This technique has been previously utilized to determine the molecular weight of glycosaminoglycans (Losito et al. 1981) and was useful to determine the molecular weight of polycomponent compounds since this procedures relies almost exclusively on size for separation. Although the elution

profiles of both heparin and CY 216 were bell-shaped, the molecular weight distribution of CY 216 was narrower than that of heparin. The frequency and molecular weight distribution averages of heparin and CY 216 were also determined. Although the molecular weight distribution profile of protamine was narrower than the heparins, the elution profile of protamine was characteristic of a compound containing more than one chemical species. Of the three compounds, CY 216 exhibited the lowest mean molecular weight (5400 daltons), followed by protamine (4,000 daltons) then heparin (11,000 daltons). Determination of the molecular weight of these agents was important in the understanding of their interactions.

D. NMR Spectra of Heparin, CY 216 and Protamine

Until recently, the complete analysis of the ^{1}H NMR spectrum of heparin has not been feasible since several signals overlap heavily even at resolutions at 270 MHz instruments. Another problem has been signal broadening due to the effect of the viscosity of the concentrated solutions used for the NMR analysis (Gatti et al. 1979). Casu and coworkers have used a resolution enhancement method which was originally utilized for the study of proteins, to assign all of the proton signals. This approach has now provided us with a complete elucidation of the structure of heparin.

In collaboration with Casu and coworkers, we have compared the ^{1}H NMR spectra of heparin and CY 216. While the NMR spectra of heparin was consistent with the known structure of heparin, CY 216 exhibited signals which differed from heparin signals. These signals of CY 216

were characteristic of the presence of anhdromannose residues and partial desulfation.

Utilizing this technique, it was demonstrated that heparin induces a spectral shift in the 3.3-3.6 ppm region after complexation with protamine. This finding has not been previously reported and requires further validation. The nature of this shift with low molecular weight heparins may provide some useful information on the interaction and binding region of heparins with protamine.

E. In Vitro Studies on Heparin and CY 216 and Their Interaction with Protamine

1. Comparison of Pooled Animal Plasma Preparations

The focus of this dissertation was to use established animal models of bleeding and thrombosis and extrapolate the results to the human plasma response. Since the animal models used in the studies incorporated three different animal species (rats, rabbits and primates) it was necessary to compare the in vitro baseline responses of the plasma preparations in the clotting and amidolytic assays to a standard normal human plasma preparation. A study of in vitro baseline responses of the plasma preparations facilitated the evaluation of the ex vivo results.

The APTT, thrombin time and Heptest^{R} activity of NratP differed significantly ($p<.05$) from NHP as determined by a one way ANOVA. The thrombin time and Heptest^{R} baseline values were elevated while the APTT baseline values were decreased as compared to NHP. The baseline clotting values for NRP were elevated as compared to NHP and the degree of this elevation was dependant on the individual clotting tests.

These differences may be related to compositional variations between the plasma preparations. The composition of NRP differs greatly from that of NHP. All coagulation factors of NRP except factor X and fibrinogen are elevated 2 to 18 fold as compared to NHP (Walenga 1987). Nonhuman primates demonstrate marked similarities to humans in almost all aspects of their anatomy, endocrinology and physiology and these similarities underlie the value of these animals for appropriate studies in neurobiology, immunology, pathology, cardiology and psychology (King et al. 1989). Evaluation of baseline clotting values confirmed reports that the clotting activity of NHP and NMP was very similar.

2. The In Vitro Effects of Protamine on Plasma

In vivo administration of protamine has been reported to influence the hemostatic system in a variety of ways. Protamine has been reported to decrease fibrinogen levels (Gans 1962; Godal 1960), aggregate platelets by direct effects (Eika et al. 1972) and produce thrombocytopenia and leukopenia (Al-Mondhiry 1985, Eika et al. 1971). However, Ellison et al. (1971) have shown that overdoses of protamine (800 mg/70 kg) had minimal effects on the coagulation mechanisms of both patients and volunteers. Therefore studies were carried out in order to determine whether protamine had any effect on the in vitro clotting tests and amidolytic assays used in these studies. This information was needed in order to evaluate the ex vivo data. After administration of 2 drugs such as heparin and protamine, it was necessary to determine the individual contribution of each drug to the prolongation of clotting time and amidolytic activity. The APTT was

significantly ($p<.05$) prolonged, (as determined by a one way ANOVA) after the in vitro supplementation of protamine (100 μg/kg i.v.) to NHP, NMP, NRP and NratP. The extent of the prolongation of the clotting times of the plasma preparations was similar.

In order to test the effect of high concentrations of protamine on the laboratory assays, protamine (1 mg/ml and 10 mg/ml) was supplemented to NHP. Protamine sulfate (1 mg/ml) prolonged the APTT to > 150 seconds, however, had no effect on the other tests. This concentration of protamine is much greater than is routinely achieved clinically. Protamine (10 mg/ml) significantly prolonged all of the clotting tests and exhibited inhibition of amidolytic anti Xa activity, however, protamine at this concentration also caused the plasma to become turbid. The effects of protamine may be nonspecific and it is unlikely that these effects of protamine occur under physiological conditions.

The APTT was most sensitive to the anticoagulant effects of protamine and was the only test which exhibited significant anticoagulant and antiprotease effects in all of the plasma preparations. The other assays were relatively insensitive to protamine.

3. In Vitro Supplementation of Heparin and CY 216 to Various Plasma Preparations

Although the constituents of primate plasma are similar to human plasma, differences between rat, rabbit and human plasma have been reported (Schalm 1975). Before beginning the animal experimentation, it was necessary to compare the in vitro heparinizability of the various animal plasma preparations in order to determine the extent the plasma differences might have on heparin and CY 216 anticoagulant and

antiprotease activities. Varying levels of endogenous neutralizing agents like PF4 can modify the activity of heparin and CY 216 to varying degrees and may influence their in vitro activity profiles in the plasma preparations.

After supplementation of heparin and CY 216 to the plasma preparations, the APTT was equally sensitive to the heparins when supplemented to NHP and NMP. The baseline APTT value for NRP was elevated as compared to the other plasma preparations therefore at CY 216 (2.5 μg/ml) the APTT was prolonged to > 150 seconds. The APTT, however, exhibited less sensitivity to heparin supplemented to NratP. In addition, since heparin must bind to AT III in order to exert anticoagulant effects, differing levels of AT III in the plasma preparations could result in variations in heparin and CY 216 activity.

Even though the APTT is less sensitive to the effects of low molecular weight heparin than to heparin, a similar pattern of clotting time elevation was seen for CY 216 and heparin after supplementation to the various plasmas. Since the APTT was relatively insensitive to the anticoagulant effects of CY 216 and it was important to determine the global clotting activity of CY 216 the Heptest^{R} was used to determine CY 216 activity in the plasma preparations. This assay which has recently developed is sensitive to the collective effects of heparins and other substances which inhibit factor Xa and factor IIa. The Heptest^{R} exhibited increased sensitivity to the effects of heparin and to CY 216 supplemented to NRP than to the other plasma preparations. The clotting assays (APTT and Heptest^{R}) were more sensitive to NRP

which may be related to increased clotting factor levels in NRP as compared to the other plasma preparation.

The % inhibition of factor X_a and factor II_a by heparin and CY 216, in general, followed a similar pattern in the various plasma preparations, however once again the response of NHP to heparin and CY 216 was most similar to the NMP response. Equigravimetric amounts of heparin exhibited greater clotting and amidolytic activity than CY 216 when supplemented in vitro to NHP, NMP, NRP and NratP.

4. In Vitro Neutralization of Heparin and CY 216 by Protamine

Although many different animal plasmas were studied in vitro, the primary objective of these studies was to relate the data obtained from the animal models to what occurs in human plasma. Therefore the in vitro neutralization of heparin and CY 216 was studied only in NHP. Although heparin and CY 216 exhibited assay dependant protamine neutralization in vitro, significant differences between the neutralization profiles of heparin and CY 216 were noted. In order to compare these differences the ratios of protamine to heparin required to completely neutralize the activity of the heparins in the individual assays were determined.

Differential neutralization of the activities of heparin and CY 216 was noted. All of the in vitro activities of heparin except amidolytic factor Xa activity were completely neutralized at a protamine to heparin ratio of 2:1. However the activities of CY 216 were neutralized to varying degrees depending upon the test. In general, the activity of CY 216 and heparin in the assays which primarily measure the inhibition of thrombin (thrombin time and amidolytic anti

IIa assays) were neutralized more readily than the assays which measure anti Xa activity (amidolytic anti Xa method and Heptest^{R}). The activity of CY 216 in the thrombin time assay was completely neutralized at a protamine to CY 216 ratio of 1:1, while the amidolytic anti Xa activity of CY 216 was neutralized by a protamine to CY 216 ratio of 2:1. The Heptest^{R} was only slightly neutralized at a protamine to CY 216 ratio of 2:1. A comparison of the protamine to heparin (or CY 216) ratios at which complete neutralization occurred for the in vitro and in vivo studies in shown in the Appendix XVIII and XIX respectively. All of the anticoagulant and antiprotease activities of heparin and all activities except for the anti Xa activity of CY 216 in the primates was neutralized at a gravimetric 1:1 ratio. The anti Xa activity of CY 216 after administration to the primates was completely neutralized by a 2:1 protamine to CY 216 ratio. In general both in vitro and in rabbits, the activity of heparin was more readily neutralizeable than CY 216.

5. In Vitro Neutralization of a Chemically Synthesized Pentasaccharide by Protamine

The in vitro neutralization of a chemically synthesized pentasaccharide by protamine has been studied by Walenga (1987). Although pentasaccharide itself is not present in CY 216, the neutralization of pentasaccharide can be considered as a good model for some ultra low moleclular weight oligosaccharides which are present in CY 216. The pentasaccharide posseses the minimum binding region of heparin which is able to induce a conformational change in AT III thus increasing the inhibitory activity of this protein towards factor Xa. However, this

activity was not neutralizeable by a 30 fold gravimetric excess of protamine. In addition, the heptest^{R} activity of pentasaccharide was neutralized approximately 60% and the anti Xa activity of pentasaccharide was neutralized approximately 90 % by polybrene. It is not known whether the lack of neutralization of the pentasaccharide by protamine is due to a lack of binding of protamine to the pentasaccharide due to molecular size or charge characteristics or whether the lack of neutralization of pentasaccharide is due to the inability of protamine to disrupt a formed pentasaccharide-AT III complex. Since polybrene was able to neutralize the anti Xa and heptest^{R} activity of the pentasaccharide more readilly than protamine, the charge density of the neutralizing agent may be important. Other structural characteristics may be required for the neutralization of the antiprotease activities of oligosaccharides. Vitronectin has been shown to efficiently neutralize oligosaccharides of M_r 2,400 - 7,200 daltons (Lane 1987).

6. In Vitro Neutralization of Heparin and CY 216 by Spermidine

Protamine has been used clinically to antagonize the hemorrhagic effects of heparin for many years and much information is known about this interaction of heparin with protamine. However, less is known about the interaction of protamine with low molecular weight heparins. The primary intent of this dissertation was to obtain practical clinically relevant information of the interaction of heparin with polybasic agents. Therefore, protamine was selected to antagonize the effects of the heparins. However it was also of interest to study the interaction of the heparins with chemically defined polybasic sub-

stances such as spermidine since this agent has a defined structure. Studies were performed on the interaction of heparin and CY 216 with spermidine to compare the *in vitro* protamine neutralization of the heparins to the spermidine neutralization profiles in order to investigate whether assay dependant neutralization of heparin and CY 216 exists when a pure substance was used as the neutralizing agent.

Spermidine was less potent as an antagonist to the anticoagulant and antiprotease effects of heparin and CY 216 than protamine. In addition, spermidine exhibited a characteristic assay dependant neutralization profile. These preliminary studies indicated that the differential neutralization of heparins after protamine and spermidine antagonism may be related to differences in their chemical structure, charge distribution or molecular weight. A comparison of the structure of spermidine and protamine is shown in Appendix XX. Protamine contains proteins of differing amino acid length which contain mostly arginine. Spermidine, on the other hand is a positively charged linear polyamine. Systematic structure activity studies are needed to further characterize the differential neutralization of the *in vitro* anticoagulant and antiprotease activities of heparin and CY 216.

F. In Vivo Studies

1. *In Vivo* Studies on the Effects of Protamine

Protamine sulfate (200 μg/kg i.v.; 2 and 3 mg/kg i.v.) was administered to rabbits in the doses which were used to antagonize heparin and CY 216 in the stasis thrombosis and the rabbit ear blood loss experiments. No significant effect of protamine on *ex vivo* clotting and antiprotease activity was seen at these doses. Similarly,

administration of protamine (2.1 mg/kg i.v.) to primates had no effect upon clotting or amidolytic anti Xa activities. Protamine exerted a significant ($p<.05$) effect on the amidolytic anti IIa assay. This may be related to a report that protamine act as a competitive inhibitor of thrombin-fibrinogens interaction and can also act as a substrate for thrombin (Cobel-Geard 1983). The observed amidolytic anti IIa activity may result from protamine acting as a secondary substrate for thrombin. Therefore less synthetic substrate is cleaved by thrombin and it appear that thrombin is inhibited after protamine administration in this assay.

It was not possible to administer increased amounts of protamine to the primates to determine whether higher doses of protamine would exert increased ex vivo anticoagulant effects since some primates did not tolerate protamine administration very well. Although protamine was administered very slowly as a bolus injection, certain primates vomited after administration of this drug. Similar reactions have been observed when other protein drugs have been administered to the primates.

2. Animal Models Used To Study The Antithrombotic Effects of Heparin and CY 216 and Protamine

Animal models of thrombosis have contributed to the current knowledge of thrombosis and have been useful in the study and development of newer therapeutic agents. In the present studies, two established animal models of thrombosis were utilized. A modified version of the classic Wessler model (Wessler 1974) of venous thrombosis and a laser-induced model of thrombosis (Weichert 1984) were used

to study the differential protamine neutralization of heparin and CY 216. Since many factors can trigger thrombosis, it was necessary to use models with different activation mechanisms in order to investigate the neutralization of the antithrombotic activity of heparins. This has provided an opportunity to determine whether similar potency and neutralization profiles are obtained when thrombosis is induced by differing mechanisms. These two thrombosis models differ in the animal species used, type of vessel under investigation, degree of blood flow, thrombogenic stimuli and method of end point detection.

Factors which promote thrombosis include: vascular damage, stimulation of platelet aggregation and intravascular activation of blood coagulation (Hirsh et al. 1983). A central hypothesis describing the initiation of thrombosis focuses on endothelial cell injury and resultant thrombus formation. Endothelial damage exposes the underlying subendothelium to platelets which adhere, release their cytoplasmic constituents and aggregate at the surface.

Although endothelial injury may result in thrombosis, it is not a prerequisite in all cases. As first described by Hewson as early as 1771, stasis alone, after a period of a several hours can induce thrombosis. However, usually a thrombogenic stimulus or challenge must be present for thrombosis to occur. Although patients are often described as being in a hypercoagulable state, the triggering mechanism for thrombosis is rarely evident. In animals and presumably in humans as well, a thrombus can be induced in an area of stasis within minutes when such agents as serum, serum factors, endotoxin and ellagic acid are present (Colman et al. 1987).

Blood flow is also an important factor in the progression of thrombogenesis. Thrombi usually do not form in normal vessels from which the endothelium has been removed if flow is laminar, however, if blood flow is disturbed materials released by adherent platelets and thrombin can accumulate in the vortices and thrombi may form.

For many years the Wessler thrombosis model has been the accepted model used in the evaluation of antithrombotic drugs. It is based on a fundamental concept of Virchow (1856) in which thrombosis is described as resulting from a hypercoagulable state, localized diminished flow (or stasis) and cellular interactions. The hypercoagulable state in the present studies was produced after the injection of PCC and RVV. PCC contains a complex of human factors II, VII, IX and X, RVV (Russell's viper venom) activates factor X to Xa. When used in combination, these agents produce high levels of factor Xa, which is thought to be the primary target of low molecular weight heparin and causes an activation of coagulation and subsequent clot formation. PCC is standardized in terms of vitamin K dependant factors, therefore the use of batches of the agents can result in reproducible results.

Localized diminished flow, the second component in Virchow's triad is achieved in the stasis thrombosis model through the use of ligatures. Following infusion, the surgically isolated jugular veins were ligated and localized stasis in addition to hypercoagulable blood, produced clot formation within the jugular veins.

Although the Wessler model is still widely used, experimental data and clinical observation has shown that endothelial damage alone is sufficient to produce thrombosis and that complete stasis is rarely

found during the progression of thrombosis in man. Therefore this model possesses certain limitations (the use complete stasis and the lack of any endothelial injury) and may not be as physiologically relevant as other models. The thrombotic stimulus in the laser-induced thrombosis model, namely, endothelial damage is thought to be a more physiologically relevant stimulus for thrombosis.

While stasis is an important parameter in the Wessler model, blood flow is an integral parameter of the laser-induced model. Complete blood flow is maintained in the vessels under investigation throughout the entire experiment and any blockage to flow which occurs is a direct result of thrombus formation. In the laser model, after the laser injury is produced any blood elements which become coagulated as a result of the laser injury are swept away by the blood flow and the several seconds elapse before the first platelets stick to the damaged vessel wall area. Therefore, it is unlikely that thrombus formation results form coagulable protein but rather forms as a result of platelets becoming activated after interaction with the subendothelium (Weichert 1983; 1984; 1986). As the experiment is carried out, blood flow carries away any platelet aggregates which initially form and break apart from the growing thrombus.

a. Antithrombotic Effects of Protamine

Protamine is administered clinically to antagonize the hemorrhagic effects which sometimes occur during antithrombotic therapy with heparins. Administration of protamine has been shown to influence coagulation proteins (Gans 1962; Godal 1960) and platelets (Eika 1972:

Al-Mondhiry 1985). It was interesting to note that protamine administration to rabbits and rats exhibited antithrombotic effects.

Protamine (100 and 740 μg/kg i.v.) produced a slight nonsignificant degree of inhibition of thrombosis in the rabbit stasis thrombosis model and the laser-induced thrombosis model respectively. Protamine was much less potent as an antithrombotic agent than the heparins in the stasis thrombosis model, however protamine was nearly as potent as heparin and CY 216 in the laser-induced thrombosis model which is very sensitive to platelet-vessel wall interactions. The antithrombotic activity of protamine in the laser model may be related to protamine effects on platelets (Al-Mondhiry 1985; Eika 1971) and inhibition of platelet-vessel wall interactions.

b. Intravenous and Subcutaneous Antithrombotic Actions of Heparin and CY 216

Equigravimetric amounts of heparin were more potent than CY 216 in both the stasis thrombosis and the laser-induced thrombosis models. However, in the stasis thrombosis model, complete neutralization of the antithrombotic effects of heparin were observed at equigravimetric amounts of protamine, while significant ($p<.05$),(as determined by a two way ANOVA) antithrombotic activity of CY 216 were observed after equigravimetric protamine. In the laser-induced thrombosis model, heparin and CY 216 were completely neutralized by protamine. The differing results between models may be related to the activation mechanisms used to induce thrombosis in the models. In the stasis model, the primary activator is targeted toward the coagulation system while, in the laser-induced thrombosis model, damaged endothelium is

the primary activator for thrombosis to occur. While in the laser model, although damaged endothelium activates platelets, fibrin helps to stabilize the platelet aggregates which form. The differential degree of neutralization of CY 216 observed in the stasis thrombosis model may be related to components within CY 216 which can still inhibit the coagulation system after protamine administration. These effects may be related to amidolytic anti Xa inhibition, however this is unlikely since the levels of amidolytic anti Xa activity were very low after the administration of CY 216 (100 μg/kg i.v.) used in the stasis thrombosis experiments. In addition, correlations between antiprotease activity and % inhibition of thrombosis were poor ranging from (.002 -.806) depending upon the assay used. Bianchini et al. (1985) found a similar lack of correlation between in vitro activity and antithrombotic effects of heparins. Perhaps some mechanism exists which is responsible for this antithrombotic activity after protamine antagonism of CY 216 which is not measurable at this time.

Significant antithrombotic activity was observed after administration of heparin (1 mg/kg s.c.) and CY 216 (1 mg/kg s.c.) to rabbits. Similarly, significant ex vivo activity was observed. The anti IIa activity of heparin and CY 216 was completely neutralized, while the anti Xa activity after subcutaneous administration of heparin and CY 216 was not neutralized. Once again there was a poor relationship between ex vivo activity and protamine neutralization of the antithrombotic activity. In the intravenous studies, CY 216 exhibited significant antithrombotic activity with slight ex vivo antiprotease activity after subcutaneous administration of CY 216. Therefore ex vivo

antiprotease activity is not the only determinant of antithrombotic activity.

3. Hemorrhagic Effects of Heparins

Many animal models have been used to determine the hemorrhagic potential of drugs. Two models used in this study, the rabbit ear blood loss model and the rat tail bleeding model, exhibit differential sensitivity to hemostatic and physiologic components including: platelets, coagulation factors, blood pressure, and smooth muscle. The differential sensitivity of the models may be related to the type of vessels which were transected during the experiments to induce blood loss.

In 1934, when heparin was being developed for clinical use, it was thought that surgical patients who would receive heparin might bleed to death. However, the administration of heparin to patients since that time without the occurrence of spontaneous hemorrhage, even during major operations has disputed this concern (Jaques 1973). For hemorrhage to occur after the administration of heparin means that several factors of hemostasis may be deranged simultaneously (Jaques 1949). Abnormalities in the normal hemostatic mechanism which can lead to bleeding include: defects in blood vessels, a deficiency in the number of circulating platelets, abnormal platelet function, defects in blood coagulation and excess fibrinolytic activity (Hirsh 1983). The hemorrhagic risk of heparin could be theoretically influenced by a number of variables including (1) the dose of heparin (2) the anti-coagulant effect of heparin as measured by a laboratory test (3) the route of heparin administration and (4) patient characteristics such as

concurrent administration of antiplatelet drugs. A commonly held clinical view is that the risk of bleeding increases with either increasing doses of heparin or with the heparin response as reflected by *in vitro* coagulation tests. Bleeding is more likely to occur when an *in vitro* test of coagulation is excessively prolonged.

In the rabbit ear model of blood loss, as developed by Cade et al. (1984), 5 uniform incisions were made in the lower portion of the rabbit ear and the incisions were standardized in such a manner that the microcapillary section of the rabbit ear was transected as shown in Appendix X. In the rat tail transection model a 2 mm section of the distal end of the tail vein was transected. These differences in methodology may lead to differing sensitivities of the models to agents which induce blood loss. The rat tail transection bleeding model is sensitive to the effect of platelets as well as coagulation factors (Dejana 1982), whereas the rabbit ear bleeding model is more sensitive to primary hemostasis (Cade et al. 1984) which involves the adherence of platelets to damaged endothelium after an injury. Heparin and low molecular weight heparin have been reported to decrease platelet adhesiveness *in vivo* which may cause a prolongation in the rat tail transection bleeding model (Harenberg 1985;Weichert and Breddin 1986).

Differences in methodology between the rat tail bleeding time and the rabbit ear blood loss model include: animal species, temperature, method of end point detection and number of determinations which can be performed per animal. Dejana et al.(1982) have demonstrated that temperature has an effect on bleeding times. Tail transection bleeding times in the rat were shown to be significantly shorter at

37°C than at 23°C. This result is in agreement with the fact that bleeding times measured in human fingers are prolonged at temperatures below 37°C (Copley 1974). One advantage of the rabbit ear blood loss model as compared to the rat tail bleeding model is that in the rabbit model temperature is more tightly controlled. In this model, the red cells are collected in a saline bath at 37° C. Also this method of end point detection is more precise than the measurement of bleeding time in the rat tail bleeding model where bleeding time was visually determined as the time when bleeding stopped. A practical advantage of the rabbit ear blood loss model is that 2 blood loss determinations can be made per rabbit (one determination on each ear) whereas only one determination can be performed on each rat.

a. Effect of Protamine in the Blood Loss Models

Protamine administration had a differential effect in the two blood loss models. The rabbit ear model of blood loss was not effected by protamine (1 and 2 mg/kg i.v.) while the rat tail bleeding model was prolonged by protamine (1 mg/kg i.v.). The prolongation in the rat tail model may be due to inhibition of primary hemostasis. Protamine has been shown to exhibit antithrombotic effects in the rat laser model of thrombosis. These antithrombotic effects of protamine may be related to inhibition of platelet adhesion and this mechanism may cause an increase in bleeding time in the rat model.

b. Intravenous and Subcutaneous Studies On the Hemorrhagic Effects of Heparin and CY 216

Good agreement between the results of blood loss induced by heparin and CY 216 was observed in the two bleeding models. Heparin (2

mg/kg i.v.) produced a significant ($p<.05$),(as determined by a one way ANOVA), amount of blood loss as compared to the control groups in both models. CY 216 produced less blood loss than heparin at equigravimetric concentrations as determined by both models. CY 216 (2 mg/kg i.v.) did not produce a significant increase in blood loss, however, the blood loss induced by CY 216 (3 mg/kg i.v.) was significant ($p<.05$) as compared to the control group, as determined by a one way ANOVA. CY 216 produced approximately 67 % of the blood loss induced by equigravimetric amounts of heparin as determined by the rabbit ear blood loss model. Low molecular weight heparins are not endowed with any special properties which would allow the clinician to ignore the dangers of hemorrhagic side effects (Thomas 1986). Since the hemorrhagic effects of heparins are dose dependant, the true efficacy of these new antithrombotic agents may not be completely realized if improper doses are administered.

Even though heparin (2 mg/kg i.v.) exhibited a greater amount of amidolytic anti Xa activity than CY 216 (2 mg/kg i.v.), the activity of heparin was more readily neutralized than the corresponding activity of CY 216. Anti Xa potency of heparins may not be directly correlated to the extent of neutralization. Differential interactions of heparin and low molecular weight heparin with protamine may be related to the size of the anticoagulant molecule. Either protamine sulfate binds to long chains more easily than to a shorter chains (Thomas 1984) or protamine may have to bind with a certain affinity to the components of heparin which inhibit factor Xa for neutralization to occur (Massonet-Castel

1986). A schematic representation of the binding of heparin to protamine is shown in the Appendix XXII.

Blood samples drawn 10 minutes after the administration of heparin or CY 216, allowed for the ex vivo analysis of blood levels of amidolytic anti Xa and amidolytic anti IIa activity at the time when the blood loss determinations were initiated. Correlations between antiprotease activity and blood loss after the administration of heparin or CY 216 were calculated. Poor correlations between ex vivo activity and blood loss were obtained for CY 216 (r=.102 for anti Xa, r=.186 for anti IIa). The correlation for Heptest^{R} and CY 216-induced blood loss was better (r=.515). It seems therefore that the hemorrhagic side effects of CY 216 are somewhat dissociated from their antiprotease activity. Similar correlations between the amidolytic anti Xa and amidolytic anti IIa activities and heparin-induced blood loss (r=.690 for anti Xa) and (r=.564 for anti IIa) were obtained.

A poor correlation between blood loss and ex vivo activity after protamine neutralization of heparin and CY 216 was seen. Heparin produced a greater amount of blood loss than CY 216 at equigravimetric doses and also exhibited greater amidolytic anti Xa and amidolytic anti IIa activity than CY 216 at equivalent doses. However, it was interesting to note that although the blood loss induced by CY 216 was completely neutralized by protamine while the amidolytic anti Xa activity of CY 216 was not. Significant amounts of amidolytic anti Xa activity of CY 216 remained after two fold gravimetric amounts of protamine was administered to rabbits.

No significant increase in blood loss was seen after subcutaneous administration of heparin (3 mg/kg s.c.) or CY 216 (3 mg/kg s.c.) to rabbits, even though significant ex vivo activity was observed in several assays. Similarly, there was no increase in blood loss after subcutaneous administration of heparin (2.5 mg/kg s.c.) or CY 216 (2.5 mg/kg s.c.) to rats. Reports have shown that bleeding occcurs more frequently when heparin is given therapeutically in large doses than when it is administered prophylactically in small doses (Colman 1987). The amidolytic anti IIa activity of subcutaneously administered heparin and CY 216 and the amidolytic anti Xa activity of heparin was completely neutralized by protamine. However, the anti Xa activity of subcutaneously administered CY 216 was not neutralized. The results correspond with the in vitro and other in vivo protamine neutralization profiles of these heparins.

4. Pharmacodynamic Effects of Heparin and CY 216 and their Modulation By Protamine

Pharmacokinetics by the strict sense of the word refers to the processes by which a drug enters and is moved around within the body (thus reaching its site of action) and is eliminated. Thus the term pharmacokinetics embraces the (1) absorption, (2) distribution and (3) elimination of drugs but not their action. Pharmacodynamics, on the other hand refers specifically to the activity of drugs (Bourne et al. 1986). Since heparin is a polycompnent drug which exerts its effects at various sites of action, it was not possible to directly measure the concentration of the active components of heparin. Therefore it was necessary to determine pharmacodynamic activities of heparin and

correlate activity data to concentration through the use of calibration curves. The pharmacokinetics of heparin are more properly termed time course of pharmacodynamic effects.

In order to directly compare the activity of heparin and CY 216 *in vivo*, a noncompartmental model was used to calculate pharmacodynamic time course parameters in primates. Noncompartmental methods for calculating pharmacodynamic time course values are based on statistical moment theory and utilize calculations of area under the concentration curve which is a simple expression of drug disposition (Gibaldi et al. 1982). To date, statistical moments have been applied to pharmacokinetic and biopharmaceutic processes dealing primarily with drug absorption and distribution. These concepts were initially used in the biomedical literature by Perl and Samuel (1969) to analyze cholesterol input and output in man and by Oppenheimer et al. (1975) in describing the disposition of iodothyronine.

Noncompartmental analysis is not "model independent" but rather is based on a model with a far more restricted structure and realm of applicability than multicompartmental models. A major conceptual advantage of a noncompartmental model is that any number of recirculations or exchanges can occur with any number of noncentral pools, none of which has to be identified with any physiological structures (Di Stefano 1984). A diagrammatic representaion of a noncompartmental model is shown in Appendix XXI.

Study of the time course of the neutralization of the heparins presented some interesting problems. It was not possible to calculate all of the pharmacodynamic time course parameters after neutralization

of the heparins by protamine since the initial heparin-protamine activity level (concentration) at time 0 must be known in order to calculate Clp and Vd. This value could not be obtained from the data since heparin was administered 5 minutes prior to protamine. The study was designed in this way in order to simulate the protamine neutralization of heparin in patients. In order to calculate Vd and Clp of heparin and protamine an in vitro heparin-protamine complex would have to be administered to the primates and the calculations determined.

a. Differential Neutralization of Heparin and CY 216 By Protamine

Assay dependant protamine neutralization after administration of heparin (0.7 mg/kg i.v.) and CY 216 (0.7 mg/kg i.v.) was observed in the primates. Both heparin and CY 216 exhibited strong anticoagulant and antiprotease effects. After the administration of protamine, the degree of neutralization of these effects varied with the laboratory assays and heparin and CY 216 exhibited characteristic neutralization profiles in the primates.

The Heptest^{R} activity of heparin was the only activity which was completely neutralized by equigravimetric protamine, while the APTT and thrombin time activities of heparin were completely neutralized at a two fold gravimetric amount of protamine. The neutralization patterns of amidolytic anti Xa and amidolytic anti IIa activity of heparin were very similar. Varying degrees of neutralization of the activities were seen up to 3 hours. The APTT, thrombin time, and anti IIa activities of CY 216 were completely neutralized by equigravimetric protamine. A dose dependant neutralization of the amidolytic anti Xa of CY 216 was seen. Complete neutralization of the amidolytic anti Xa activity of CY

216 was not observed at three fold gravimetric concentrations of protamine. Virtually no neutralization of the Heptest^{R} activity of CY 216 was seen after administration of three fold gravimetric amounts of protamine.

b. Intravenous Studies

Protamine neutralization of the pharmacodynamic time course of intravenously administered heparin and CY 216 was studied in the primate (macaca mulatta). Activity of heparin and CY 216 was determined using APTT, thrombin time, Heptest^{R}, amidolytic anti Xa and amidolytic anti IIa methods. Pharmacodynamic time course parameters were calculated from concentrations obtained from Heptest^{R} and amidolytic anti Xa activity data which was extrapolated from calibration curves of heparin or CY 216 supplemented to NMP. It was not possible to calculate pharmacodynamic time course parametres for the anti IIa data since a large proportion of the data points were out side of the linear range. Also after protamine administration, the anti IIa activity of the heparins was completely neutralized therefore it was not possible to calculate area under the curve with only a few data points. To determine the anti IIa time course parameters, it would be necessary to use lower doses of the heparins and draw blood more frequently.

1. Area Under the Concentration Curve (AUC)

Increasing the doses of heparin and CY 216 administered to the primate groups from 0.35 mg/kg i.v. to 0.7 mg/kg i.v. resulted in a dose dependant increase in AUC values. In most cases as the doses doubled the AUC values increased more than two fold. This is related

to decreases in the plasma clearance which were observed as the dose of heparin was increased as determined by the amidolytic anti Xa and Heptest^{R} methods. Similar findings have been reported by Worth and Estes (1969) and Bjornsson et al. (1981).

In general, the differences between the time course parameters of heparin and CY 216 lessened as the dose was increased to 0.70 mg/kg i.v. At lower doses, differences between drugs may be more pronounced since drug elimination mechanisms do not become saturated and dose-dependant pharmacodynamics are not apparent at lower doses. The AUC for CY 216 as determined by the amidolytic anti Xa method did not increase proportionately with the increase in dose. This may be related to the plasma clearance of CY 216. The Cl_p for CY 216 as measured by the amidolytic anti Xa method was not dose related, while the Cl_p decreased with increasing dose as determined by the Heptest^{R}.

From the AUC values of heparin and CY 216 after protamine administration it was apparent that both heparin and CY 216 demonstrated characteristic protamine neutralization profiles as determined by the amidolytic anti Xa assay and Heptest^{R}. Comparison of AUC values provided a means of quantitating the activity and degree of neutralization of the heparins over an extended period of time rather than at one certain time period. Both assays demonstrated that the ex vivo activity of CY 216 was less neutralizable by protamine than the ex vivo activity of heparin.

2. Mean Residence Time (MRT)

Increasing the doses of heparin administered to the primates from 0.35 mg/kg i.v. to 0.7 mg/kg i.v. resulted in a significant ($p<.05$)

dose dependant increase in the MRT as determined by the Heptest^{R} but not the amidolytic anti Xa method. Once again differences in sensitivity of the assays may lead to different pharmacodynamic time course parameters. Differences in MRT values could also be related to intergroup differences within the primate groups. Increasing the dose of CY 216 from 0.35 mg/kg i.v. to 0.7 mg/kg i.v., however, resulted in a significant ($p<.05$) decrease in MRT values as determined by both methods. Protamine (0.7, 1.4 or 2.1 mg/kg i.v.) administration did not have any significant effect on the MRT valued of heparin or CY 216 determined by both methods.

3. Plasma Clearance (Clp)

Increasing the dose given to the primates from 0.35 to 0.70 mg/kg i.v. significantly ($p<.05$) decreased the clearance of heparin as determined by both methods. In contrast, no significant change in the Cl_p of CY 216 was noted. This may be related to the differing route and kinetics involved in their elimination of heparin and CY 216. Heparin is primarily cleared by the liver and kidneys (Palm and Mattson 1987) from a combination of saturable and nonsaturable mechanisms. (DeSwart 1982) The saturable mechanism of heparin clearance involves binding to the endothelium (Dawes and Pepper 1979) while the nonsaturable mechanisms may involve renal elimination (Piper 1947). Low molecular weight heparins, on the other hand, are cleared primarily via the kidneys through renal filtration (Goudable et al. 1986). Certain routes of elimination such as endothelial cell binding may become saturated for heparin and renal and hepatic elimination may predominate and as a result, clearance may increase with increasing dose.

4. Apparent Volume of Distribution (Vd)

A significant ($p<.05$) dose dependant decrease in the Vd of heparin was observed as the dose of heparin was doubled as determined by the amidolytic anti Xa method but not as determined by the Heptest^R. Intuitively one would not expect the Vd to decrease as the dose increased. This anomalous result was not found with the Heptest^R, which suggest that an assay which measures the collective activities of heparin may be a more reliable method for determining heparin pharmacodynamic time course parameters than a specific assay. Although both Vd values for heparin and CY 216 < 0.05 l/kg which indicated that the drugs remained within the plasma compartment. The Vd of heparin was generally greater than for CY 216 which may be related to the increasing endothelial affinity of heparin with increasing molecular weight. No significant dose related change in the Vd of CY 216 was noted by either method.

5. Protamine Neutralization Index (PNI)

PNI values were calculated for intravenously administered heparin and CY 216 after neutralization by protamine. These values provided a means of quantitating the degree of neutralization of the drugs over an extended time period. PNI values were generally greater for heparin than for CY 216 as determined by both methods, however no difference was found between the PNI values of heparin and CY 216 at a gravimetric protamine to heparin (or CY 216) ratio of 1. A greater PNI value was observed at a gravimetric protamine to heparin ratio of 3:1 as determined by the amidolytic anti Xa method.

c. Subcutaneous Administration

Protamine neutralization of the pharmacodynamic time course of subcutaneously administered heparin and CY 216 was studied in the primate (macaca mulatta). Pharmacodynamic time course parameters were calculated from concentrations obtained from Heptest^{R} and amidolytic anti Xa activity data which was extrapolated from calibration curves of heparin or CY 216 supplemented to NMP.

1. Area Under the Concentration Curve (AUC)

The AUC values for CY 216 (0.7 mg/kg s.c.) were increased as compared to heparin (0.7 mg/kg s.c.). Since the degree of absorption after subcutaneous administration of a drug depends upon molecular weight, greater antiprotease activity was observed after subcutaneous administration of CY 216. Heparin exhibits an increased mean molecular weight as compared to CY 216 and was not substantially absorbed after subcutaneous administration. After protamine administration to heparin (0.7 mg/kg s.c.) or CY 216 (0.7 mg/kg s.c.) a decrease in the AUC values were observed as determined by the amidolytic anti Xa method but not the Heptest^{R}.

2. Mean Residence Time (MRT)

The MRT of CY 216 (0.7 mg/kg s.c.) was longer than the MRT of heparin (0.7 mg/kg s.c.) as determined by the amidolytic anti Xa method. This is consistent with literature reports (Palm and Mattson 1987; Lockner et al. 1986). It was interesting to note that the MRT values for CY 216 (0.7 mg/kg s.c.) were not greater than the MRT for heparin (0.7 mg/kg s.c.) as determined by the Heptest^{R}. However this result may be related to the large standard errors which were obtained for the MRT values after subcutaneous administration of heparin and CY

216. There was no significant effect of protamine on the MRT values of CY 216 (0.7 mg/kg s.c.) as determined by both methods, or for heparin (0.7 mg/kg s.c.) as determined by the amidolytic anti Xa method. However the MRT for heparin (0.7 mg/kg s.c.) was significantly ($p<.05$) less after the administration of protamine as determined by the Heptest^R.

3. Plasma Clearance (Clp)

The plasma clearance values obtained after subcutaneous administration of heparin (0.7 mg/kg) were increase as compared to heparin (0.7 mg/kg i.v.). The Clp of CY 216 (0.7 mg/kg s.c.) was increased as compared to CY 216 (0.7 mg/kg i.v.) as determined by the Heptest^R and amidolytic anti Xa methods. The clearance values were dependant upon the method of calculation used in the noncompartmental model. Clearance was calculated as Cl_p = (dose)/(AUC), however the absorption of heparin was very poor. Therefore the clearance values calculated by this method reflect the poor absorptive characteristics of heparin after subcutaneous administration rather than the actual clearance. The plasma clearance for CY 216 as determined by the Heptest^R was greater than as determined by the amidolytic anti Xa method. Once again this result may be related to the absorptive characteristics of CY 216 and the sensitivity of the assay. Heptest^R measures both low molecular weight components which inhibit factor Xa and high molecular weight components which may inhibit factors Xa and IIa. Since high molecular weight components are not absorbed as well as low molecular weight components, it may appear that more heparin molecules are cleared while actually they may not have been absorbed.

4. Apparent Volume of Distribution (Vd)

The Vd values for heparin (0.7 mg/kg s.c.) and CY 216 (0.7 mg/kg s.c.) were low (<0.05 l/kg) which indicates after absorption, the drugs remain in the plasma compartment. The Vd for CY 216 (0.7 mg/kg s.c.) was 10 fold lower than the Vd for heparin as determined by both methods and the Vd for CY 216 (0.7 mg/kg s.c.) as determined by the Heptest^{R} The Vd approximates the apparent volume into which a drug distributes and is not a real volume. Low values for Vd may have been obtained since the equation used to calculate Vd:

$$Vd = \frac{\text{dose x AUC}}{(\text{AUMC})(\text{AUMC})} \times \frac{\text{1 liter}}{\text{1000 ml}}$$

is inversely related to the AUMC. This value which is related to the AUC was elevated 3 fold as compared to the AUMC for CY 216 (0.7 mg/kg s.c.) as determined by the Heptest^{R}. Once again differing sensitivities of the assays contributed to variations in the calculated parameters.

5. Protamine Neutralization Index (PNI)

PNI values were calculated for subcutaneously administered heparin and CY 216 after neutralization by protamine. The activities of CY 216 were less neutralizeable than heparin as determined by the amidolytic anti Xa method as compared to the Heptest^{R}. It is interesting to note that a lesser degree of neutralization after subcutaneous administration of both drugs occurred as compared to intravenous administration. The activity of the drugs was neutralized approximately 50 % less after subcutaneous administration.

d. Practical Implications of Protamine Interactions with Heparin and CY 216

1. The Relevance of Laboratory Tests in the Assessment of Heparin and CY 216 After Neutralization By Protamine

Very limited comparative information on the interaction of protamine with heparin and low molecular weight heparins is available. The information which is available is limited in that it does not address the practical considerations of the interaction of protamine with low molecular weight heparin such as the relevance of ex vivo activity of low molecular weight heparins to thrombosis or bleeding after neutralization by protamine or low molecular weight heparin rebound.

The current studies have addressed the relevance of ex vivo activity as determined by anticoagulant and antiprotease assays to bleeding and antithrombotic effects. These tests may have some limited value in the prediction of which patients will exhibit blood loss after the administration of heparin or low molecular weight heparin. After heparin administration to rabbits in the blood loss studies, the anti Xa (r=.690) and anti IIa (r=.564) correlation coefficients were similar as well as the correlation coefficient obtained after administration of CY 216 to rabbits as determined by the Heptest^{R} (r=.515). However the correlation coefficient for CY 216 as determined by the amidolytic anti Xa (r=.102) and anti IIa (r=.186) methods were poor.

After protamine neutralization, the thrombin time exhibited the best correlation coefficients (r=.765 for heparin), (r= .812 for CY 216) for blood loss and activity . The use of particular laboratory tests to measure heparin and low molecular weight heparin activity may not necessarily be sensitive to the effects of the heparins after

protamine neutralization. Although the studies were limited, the data suggests that of the tests used, either amidolytic anti Xa or amidolytic anti IIa assay would be useful to predict the hemorrhagic effects of heparin however these tests do not predict the hemorrhagic potential of CY 216 as well as the Heptest^{R}. After protamine neutralization of heparin and CY 216, the thrombin time assay best correlated with increased blood loss. These results demonstrate an important consideration in the clinical monitoring of patients who receive heparins.

a. Heparin Rebound

Many factors may contribute to the phenomenon of heparin rebound which include: the recirculation of heparin (Gollub 1967), hypothermia (Gollub 1967) and the protamine to heparin ratio administered (Kesteven et al. 1986). Although the whole blood activated clotting time is widely used to predict the neutralizing dose of protamine sulfate (Piffare et al. 1981), this assay may not always give a true reflection of the dose of protamine required to neutralize heparin (Esposito et al. 1983). One objective of this dissertation was to investigate whether the individual activities of heparin and CY 216 exhibited any characteristic rebound patterns in the primate pharmacodynamic model. At these doses studied, no significant degree of heparin rebound was observed although a slight decrease in thrombin time was observed after the administration of protamine. The neutralization of this activity may appear as a slight degree of rebound, however protamine is known to have fibrinoplastic effects (Hougie 1958) which may be related to the shortening of the thrombin time and this apparent rebound of thrombin

time activity No physical signs of heparin rebound such as increased blood loss were observed in the primates.

b. Clinical Indications for Low Molecular Weight Heparins

In certain clinical conditions, such as atherosclerosis and diabetes (O Brien 1984) and during procedures such as cardiopulmonary bypass and renal dialysis, platelets become activated and release PF4. Since platelet factor 4 is known to neutralize the effects of heparins in a similar manner to protamine, (Racanelli 1987), it may be advantageous for low molecular weight heparin to retain antithrombotic activity and amidolytic anti Xa activity after complexation with polybasic proteins. This dissociation of the antithrombotic and hemorrhagic activity of low molecular weight heparins after protamine reversal is a unique property not found in heparin.

c. Future Studies

The current studies have addressed such practical issues as the relevance of anticoagulant and antiprotease activity of heparins after neutralization by protamine, however many areas concerning the interaction of heparins have not been addressed. Further studies are required to better understand the interaction of these drugs. Systematic structure activity studies are needed to characterize the interaction of heparins and protamine on the molecular level. The role of protamine neutralization of low molecular weight heparins as determined by newly developed assays which measure thrombin antithrombin complex formation or thrombin generation may provide additional insight into the exact mechanism of protamine antagonism of heparins. More extensive studies on the rebound of low molecular weight heparins after

protamine administration should be carried out to see if these agents exhibit rebound phenomenon in a way analogous to heparin. Also the hemodynamic effects of the protamine reversal of low molecular weight heparin therapy should be addressed. Protamine antagonism of low molecular weight may provide therapeutic benefits as compared to the protamine antagonism of heparin.

CHAPTER VI

SUMMARY AND CONCLUSIONS

Significant differences between the protamine neutralization of heparin and CY 216 have been observed both in vitro and in animal models of hemorrhage and thrombosis.

1. The APTT was more sensitive to in vitro supplementation of protamine than the thrombin time, Heptest^R, amidolytic anti Xa or amidolytic anti IIa assays.

2. NHP and NMP exhibited similar activity in the clotting tests, amidolytic anti Xa and amidolytic anti IIa assays after supplementation of heparin or CY 216 as compared to NRP and NratP.

3. The in vitro neutralization profiles of heparin and CY 216 by protamine differed significantly and demonstrated assay dependant characteristics. Most activities of heparin were completely neutralized at an equigravimetric protamine concentration, while the amidolytic anti IIa and thrombin time activities of CY 216 were neutralized to a greater extent than the amidolytic anti Xa and Heptest^R activities.

4. Administration of protamine sulfate (0.74 mg/kg i.v.) resulted in antithrombotic activity in the laser-induced thrombosis model and rabbit stasis thrombosis model.

5. Both heparin and CY 216 exhibited significant inhibition of thrombosis in the laser-induced thrombosis model and the rabbit stasis thrombosis model. At concentrations of 25 μg/ml and 50 μg/ml, heparin was

more potent as an antithrombotic agent than equivalent gravimetric amounts of CY 216.

6. Protamine neutralized the antithrombotic activity of intravenously administered heparin and CY 216 in the laser-induced thrombosis model to a similar extent, however, the heparins exhibited differential neutralization in the rabbit stasis thrombosis model. The antithrombotic activity of heparin was completely neutralized at a gravimetric protamine to heparin ratio of 1:1, while CY 216 (100 μg/kg i.v.) exhibited significant ($p<.05$) antithrombotic activity even after the administration of equigravimetric protamine.

7. Poor correlations coefficients were obtained between intravenously administered heparin and % of thrombosis in a rabbit stasis thrombosis model. The correlation coefficients ranged from .002 for the APTT to .515 for the amidolytic anti Xa assay. Similarly, correlation coefficients were obtained for CY 216 and % inhibition of thrombosis which ranged from .152 for the APTT to .806 for the Heptest^{R}.

8. Heparin and CY 216 (1 mg/kg s.c.) exhibited significant antithrombotic activity in the rabbit stasis thrombosis model. This activity of heparin was completely neutralized by protamine (1 mg/kg i.v.) while the antithrombotic activity of CY 216 was not completely neutralized by equigravimetric protamine.

9. Protamine sulfate (2 and 3 mg/kg i.v.) had no effect on blood loss in the rabbit ear model while an equivalent amount of protamine produced an in increase in bleeding time in the rat tail transection bleeding model. The rat tail transection data was associated with large

standard deviations. In addition, protamine administration alone caused a prolongation of bleeding time which makes the interpretation of these experiments difficult.

10. CY 216 produced approximately 60% less blood loss as equigravimetric amounts of heparin as determined by a rabbit ear blood loss model. The blood loss induced by CY 216 exhibited a poor correlation to amidolytic anti Xa (r=.102) and amidolytic anti IIa (r=.186) activity. CY 216 induced blood loss demonstrated a better correlation to the Heptest^{R} (r=.515). Heparin-induced blood loss exhibited poor correlations to amidolytic anti Xa (r=.690) and amidolytic anti II (r=.564) activity.

11. Subcutaneous administration of heparin or CY 216 (3 mg/kg s.c.) did not produce an increase in blood loss in the rabbit ear model. A similar result was obtained in the rat tail transection model where heparin or CY 216 (2.5 mg/kg s.c.) did not increase the bleeding time after tail transection.

12. The differential neutralization observed for heparin and CY 216 in vitro was observed in vivo as well. The anticoagulant and antiprotease activities of heparin were neutralized to a greater degree than the similar activities of CY 216. In general, assays which measure the anti Xa activity of CY 216 were less neutralizeable than assays which measure thrombin inhibition.

13. After intravenous and subcutaneous administration of equigravimetric doses of heparin and CY 216 to primates, the AUC of CY 216 was greater than for heparin as determined by the amidolytic anti Xa assay and Heptest^{R}.

14. In the intravenous studies the anticoagulant and antiprotease activities of heparin were more readily neutralized by protamine than the activities of CY 216.

15. Neutralization after subcutaneous administration of heparin and CY 216 was dependant upon the bioavailability and time course of the drugs. In general, heparin was neutralized more readily than CY 216.

16. A greater dose dependant decrease in the AUC of heparin was seen after increasing doses of protamine were administered. Above a protamine to heparin (or CY 216) ratio of 2:1, the PNI of heparin was significantly greater than the PNI value for CY 216.

TABLE 1

BASELINE VALUES OF NHP, NMP, NRP AND NratP

Test	NHP	NMP	NRP	NratP
APTT (seconds)	30.5 ± 2.2	24.4 ± 0.7	56.6 ± 0.8*	16.1 ± 0.9*
Thrombin Time (5 U/ml) (seconds)	21.1 ± 0.9	31.3 ± 3.6	42.3 ± 2.9*	>150
Heptest^{R} (seconds)	15.5 ± 1.2	20.5 ± 1.7	25.3 ± 0.6	56.6 ± 0.8*
Anti Xa (% Inhibition)	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Anti IIa (% Inhibition)	0 ± 0	0 ± 0	0 ± 0	0 ± 0

Data represents a mean ± S.E. N=3. Statistical Analysis was performed for each laboratory test by a one-way ANOVA, followed by a Newman-Keuls multiple comparison test. * represents a significant ($p < .05$) difference from NHP.

Table 2. Data represents a mean ± S.E., N=3.
Statistical analysis was performed for each laboratory test by a one way ANOVA, followed by a Newman-Keuls multiple comparison test.
* represents a significant difference ($p < .05$) from NHP.
** Protamine caused turbidity in the solution which may be related to the experimental result.
N.D. = not determined.

TABLE 2
IN VITRO SUPPLEMENTATION OF PROTAMINE TO
NHP, NMP, NRP AND NratP

Test	Protamine Concentration	NHP	NMP	NRP	NratP
APTT (seconds)	0 μg/ml	30.5 ± 2.2	24.4 ± 0.7	56.6 ± 0.8*	16.1 ± 0.9
	10 μg/ml	38.9 ± 1.2	26.5 ± 0.7	96.5 ± 8.3*	15.6 ± 1.1
	100 μg/ml	50.2 ± 0.6	51.1 ± 3.7	154.0 ± 15.3*	29.5 ± 5.2
	1 mg/ml	>150	N.D.	N.D.	N.D.
	10 mg/ml	>150	N.D.	N.D.	N.D.
Thrombin Time (5 U/ml) (seconds)	0 μg/ml	21.1 ± 0.9	31.3 ± 3.6	42.3 ± 2.9*	>150
	10 μg/ml	24.7 ± 0.7	34.6 ± 4.3	30.7 ± 3.6	>150
	100 μg/ml	23.8 ± 1.9	33.8 ± 1.9	32.3 ± 3.7	>150
	1 mg/ml	19.7 ± 0.8	N.D.	N.D.	N.D.
	10 mg/ml	>150	N.D.	N.D.	N.D.
Heptest^{R} (seconds)	0 μg/ml	15.5 ± 1.2	20.5 ± 1.7	25.3 ± 0.6	56.6 ± 0.8*
	10 μg/ml	18.0 ± 0.2	23.1 ± 1.2	28.9 ± 1.2	63.3 ± 0.8*
	100 μg/ml	23.0 ± 0.5	25.7 ± 4.0	28.3 ± 2.5	56.2 ± 3.1*
	1 mg/ml	27.3 ± 2.8	N.D.	N.D.	N.D.
	10 mg/ml	>300	N.D.	N.D.	N.D.
Anti Xa (% Inhibition)	0 μg/ml	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	10 μg/ml	4.6 ± 3.3	0 ± 0	0 ± 0	0 ± 0
	100 μg/ml	0 ± 0	2.0 ± 2.0	2.5 ± 1.5	0 ± 0
	1 mg/ml	1 ± 1**	N.D.	N.D.	N.D.
	10 mg/ml	53.3 ± 3.0	N.D.	N.D.	N.D.
Anti IIa (% Inhibition)	0 μg/ml	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	10 μg/ml	0 ± 0	1.2 ± 0	0 ± 0	2.6 ± 1.5
	100 μg/ml	6.4 ± 3.8	0 ± 0	0.3 ± 0.3	5.0 ± 1.8
	1 mg/ml	0 ± 0	N.D.	N.D.	N.D.
	10 mg/ml	0 ± 0	N.D.	N.D.	N.D.

TABLE 3

IN VITRO NEUTRALIZATION OF HEPARIN BY SPERMIDINE

	APTT (seconds)	Thrombin Time (seconds)	Heptest (seconds)	Anti Xa % I	Anti IIa % I
NHP	30.1 ± 0.3	22.3 ± 4.8	15.4 ± 0.1	0 ± 0	0 ± 0
NHP + Spermidine (10 Mg/ml)	36.2 ± 0.3	20.6 ± 0.3	16.7 ± 0.4	0 ± 0	0 ± 0
Heparin (5.0 Mg/ml)	>150	>150	115.2 ± 5.8	44.0 ± 1.5	81.0 ± 1.1
Heparin (5.0 Mg/ml and Spermidine (2.5 Mg/ml)	>150	>150	108.9 ± 2.2	52.7 ± 0.6	85.0 ± 1.0
Heparin (5.0 Mg/ml) and Spermidine (5.0 Mg/ml)	>150	>150	111.3 ± 3.9	49.5 ± 1.6	81.0 ± 3.3
Heparin (5.0 Mg/ml and Spermidine (10.0 Mg/ml)	>150	>150	106.2 ± 5.3	49.0 ± 4.4	67.3 ± 3.2
Heparin (5.0 Mg/ml) and Spermidine (1.0 mg/ml)	109.3	>150	95.7	66	54

Data represents a mean ± S.E., N=3, (except for heparin and spermidine 1.0 mg/ml) where data has an N=1.

TABLE 4

IN VITRO NEUTRALIZATION OF
CY 216 BY SPERMIDINE

	APTT (seconds)	Thrombin Time (seconds)	Heptest (seconds)	Anti Xa % I	Anti IIa % I
NHP	30.1 ± 0.3	22.3 ± 0.1	15.4 ± 0.1	0 ± 0	0 ± 0
NHP + Spermidine	36.2 ± 0.3	20.6 ± 0.2	16.7 ± 0.4	0 ± 0	0 ± 0
CY 216 (5.0 Mg/ml)	48.0 ± 2.4	>150	68.9 ± 5.7	38.5 ± 2.6	30.8 ± 3.2
CY 216 (5.0 Mg/ml) and Spermidine (2.5 Mg/ml)	47.2 ± 2.4	>150	46.2 ± 4.1	14.0 ± 1.8	2.7 ± 0.4
CY 216 (5.0 Mg/ml) and Spermidine (5.0 Mg/ml)	46.4 ± 2.3	>150	72.4 ± 1.7	15.7 ± 1.2	8.0 ± 0.1
CY 216 (5.0 Mg/ml) and Spermidine (10 Mg/ml)	44.7 ± 0.7	>150	64.1 ± 4.6	17.3 ± 2.9	0.3 ± 0.0
CY 216 (5.0 Mg/ml) and Spermidine (1 mg/ml)	36.8	148	43.8	45	0

Data represents a mean ± S.E., N = 3, (except for CY 216 and spermidine 1.0 mg/ml) where data has an N = 1.

TABLE 5

COAGULATION PARAMETERS OF RABBITS ADMINISTERED INTRAVENOUS PROTAMINE SULFATE

Treatment	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^{R} (seconds)	Anti Xa (% I)	Anti IIa (% I)
Saline Control	83.9 ± 1.0	32.0 ± 4.1	30.8 ± 3.8	0 ± 0	0 ± 0
Protamine 100 Mg/kg i.v.	102.3 ± 9.0	35.3 ± 3.3	28.7 ± 4.2	0 ± 0	0 ± 0
Protamine 500 Mg/kg i.v.	97.6	45.6	41.3	0	0

Saline or protamine was administered to groups of rabbits (N=5) and allowed to circulate for 10 minutes. Protamine (500 ug/kg i.v.) group contained 2 rabbits. Blood was drawn by cardiac puncture just prior to thrombogenic challenge. APTT, thrombin time, Heptest^{R}, amidolytic anti Xa and amidolytic anti IIa assays were performed. The results represent a mean ± S.E. Statistical analysis was performed for the APTT results by an unpaired t-test (two tailed), t=1.649, df=6, p >.20, N.S.

TABLE 6

COAGULATION PARAMETERS OF RABBITS
ADMINISTERED INTRAVENOUS HEPARIN

Treatment	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^R (seconds)
Saline Control	83.9 ± 1.0	32.0 ± 4.1	30.8 ± 3.8
Heparin 25 Mg/kg i.v.	124.7 ± 13.2	46.7 ± 4.4	44.9 ± 9.7
Heparin 50 Mg/kg i.v.	111.2 ± 17.2	132.3 ± 57.1*	76.8 ± 10.6
Heparin 100 Mg/kg i.v.	146.8 ± 29.7*	>150	128.9 ± 27.7*

Saline or protamine was administered to groups of rabbits (N=5) and allowed to circulate for 10 minutes. Blood was drawn by cardiac puncture just prior thrombogenic challenge. APTT, thrombin time, and Heptest^R were performed. The results represent the mean ± S.E. Statistical analysis was performed by a one-way ANOVA.

for APTT, $F(3,16)=5.847$ $p < .05$

for thrombin time, $F(3,16)=7.028$ $p < .05$

for Heptest^R, $F(3,16)=5.352$ $p < .05$

Newman-Keuls multiple comparison tests were also performed.

* represents a significant difference from the control group

TABLE 7

COAGULATION PARAMETERS OF RABBITS
ADMINISTERED INTRAVENOUS CY 216

Treatment	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^R (seconds)
Saline Control	83.9 ± 1.0	32.0 ± 4.1	30.8 ± 3.8
CY 216 25 Mg/kg i.v.	58.0 ± 6.4	37.0 ± 1.6	42.4 ± 4.1
CY 216 50 Mg/kg i.v.	49.1 ± 14.4	50.7 ± 9.3*	42.1 ± 2.1
CY 216 100 Mg/kg i.v.	67.7 ± 12.9	49.1 ± 10.7*	63.6 ± 11.8*

Saline or protamine was administered to groups of rabbits (n=5) and allowed to circulate for 10 minutes. Blood was drawn by cardiac puncture just prior to thrombogenic challenge. APTT, Thrombin time, and Heptest^R were performed. The results represent the mean ± S.E. Statistical analysis was performed by a one way ANOVA for each individual assay followed by a Newman-Keuls multiple comparison test.

for APTT; $F(3,12)=.468$ $p > 50$, N.S.

for thrombin time: $F(3,12)=19.860$, $p < .05$

for Heptest^R: $F(3,12)=5.488$, $p < .05$

* represents a significant difference from the control group.

Table 8. Saline, heparin or CY 216 was administered to groups of rabbits (N=5) and allowed to circulate for 5 minutes at which time protamine was administered to groups which received heparin or CY 216. Blood was drawn 5 minutes after protamine administration or 10 minutes after saline administration for the control group. A thrombogenic challenge was administered immediately after blood draw. APTT, thrombin time, and Heptest^{R} were performed. The results represent the mean ± S.E. Statistical analysis was performed by a two-way ANOVA.

for APTT
Factor A (drug treatment):$F(2,36)=2.655$ $p > .10$ N.S.
Factor B (protamine concentration):$F(2,36)=4.365$ $p < .05$
Factor A x B:$F(4,36)=3.288$ $p > .10$ N.S.

for thrombin time
Factor A (drug treatment):$F(2,36)=55.707$ $p < .05$
Factor B (protamine concentration):$F(2,36)=65.638$ $p < .05$
Factor A x B:$F(4,36)=56.264$ $p < .001$

for Heptest^{R}
Factor A (drug treatment):$F(2,36)=7.142$ $p < .05$
Factor B (protamine concentration):$F(2,36)=7.443$ $p < .05$
Factor A x B:$(4,36)=4.198$ $p > .05$ N.S.

Newman-Keuls multiple comparison tests were also performed.

* represents a significant difference from the control group.

** represents a significant difference from the heparin (100 μg/kg i.v.) and protamine (100 μg/kg i.v.) group.

*** represents a significant difference from the CY 216 (100 μg/kg i.v.) and protamine (100 μg/kg i.v.) group.

TABLE 8

COAGULATION PARAMETERS OF RABBITS ADMINISTERED INTRAVENOUS HEPARIN OR CY 216 AND PROTAMINE

Treatment	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest® (seconds)
Saline Control	83.9 ± 1.0	32.0 ± 4.1	30.8 ± 3.8
Heparin 100 Mg/kg i.v.	146.8 ± 29.7	>150	128.9 ± 27.7*
Heparin 100 Mg/kg i.v. and Protamine 100 Mg/kg i.v.	57.6 ± 10.2	40.3 ± 5.2	37.2 ± 6.8
Heparin 100 Mg/kg i.v. and Protamine 200 Mg/kg i.v.	122.9 ± 12.3**	37.5 ± 1.9	47.0 ± 2.0
CY 216 100 Mg/kg i.v.	67.7 ± 12.9	49.1 ± 10.7*	63.6 ± 11.8*
CY 216 100 Mg/kg i.v. and Protamine 100 Mg/kg i.v.	58.0 ± 13.6	34.1 ± 3.4	46.2 ± 4.1
CY 216 100 Mg/kg i.v. and Protamine 200 Mg/kg i.v.	113.0 ± 6.6***	38.4 ± 3.1	43.4 ± 1.3

Table 9. Saline, heparin or CY 216 was administered to groups of rabbits (N=5) and allowed to circulate for 5 minutes at which time protamine was administered to groups which received heparin or CY 216. Blood was drawn 5 minutes after protamine administration or 10 minutes after saline administration for the control group. Thrombogenic challenge was administered immediately after blood draw. APTT, thrombin time, and Heptest^{R} were performed. The results represent the mean ± S.E. Statistical analysis was performed by a two-way ANOVA.

for APTT
Factor A (drug treatment):$F(2,24)=1.032$ $p > .50$ N.S.
Factor B (protamine concentration):$F(1,24)=.33$ $p > .50$ N.S.
Factor A x B:$F(2,24)=2.793$ $p > .10$ N.S.

for thrombin time
Factor A (drug treatment):$F(2,24)=12.245$ $p < .05$
Factor B (protamine concentration):$F(1,24)=2.401$ $p < .05$
Factor A x B:$F(2,24)=1.061$ $p > .50$ N.S.

for Heptest^{R}
Factor A:$F(2,24)=2.999$ $p > .10$ N.S.
Factor B:$F(1,24)=.184$ $p > .50$ N.S.
Factor A x B:$F(2,24)=1.982$ $p > .20$ N.S.

Newman-Keuls multiple comparison tests were also performed.
* Represents a significant difference from the control group.

TABLE 9

COAGULATION PARAMETERS OF RABBITS ADMINISTERED SUBCUTANEOUS HEPARIN OR CY 216 AND PROTAMINE

Treatment	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest[R] (seconds)
Saline Control	83.9 ± 1.0	32.0 ± 4.1	30.8 ± 3.8
Heparin 1 mg/kg s.c.	61.0 ± 10.5	128.8 ± 13.4*	62.4 ± 12.4*
Heparin 1 mg/kg s.c. and Protamine 1 mg/kg i.v.	78.8 ± 18.7	85.9 ± 25.1*	39.5 ± 5.7*
CY 216 1 mg/kg s.c.	95.4 ± 4.4	54.8 ± 8.9	42.7 ± 12.2*
CY 216 1 mg/kg s.c. and Protamine 1 mg/kg i.v.	73.7 ± 12.0	37.8 ± 5.2	58.4 ± 10.2*

TABLE 10

COAGULATION PARAMETERS AFTER INTRAVENOUS PROTAMINE ADMINISTRATION IN RABBITS

Treatment (N)	Thrombin Time (5 U/ml) (seconds)	Heptest^{R} (seconds)	Anti Xa (% I)	Anti IIa (% I)
Saline Control (5)	30.1 ± 3.3	24.8 ± 3.0	0 ± 0	0 ± 0
Protamine 2 mg/kg i.v. (4)	37.0 ± 1.5	26.4 ± 3.7	2.0 ± 1.7	0 ± 0
Protamine 3 mg/kg i.v. (3)	33.2 ± 7.3	22.4 ± 0.7	0 ± 0	0 ± 0

Saline or protamine was administered to rabbits and allowed to circulate for 10 minutes. Blood was drawn by cardiac puncture just prior to blood loss determination. Thrombin time, Heptest^{R}, amidolytic anti Xa and amidolytic anti IIa assays were performed. The results represent a mean ± S.E.

TABLE 11

COAGULATION PARAMETERS AFTER INTRAVENOUS HEPARIN ADMINISTRATION IN RABBITS

Treatment (N)	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^{R} (seconds)
Saline Control (5)	83.2 ± 11.5	30.1 ± 3.3	24.8 ± 3.0
Heparin 1 mg/kg i.v. (6)	>150	>300	>271.5
Heparin 2 mg/kg i.v. (14)	>150	>300	>300

Saline or heparin was administered to rabbits and allowed to circulate for 10 minutes. Blood was drawn by cardiac puncture just prior to blood loss determination. Thrombin time, Heptest^{R}, amidolytic anti Xa and amidolytic anti IIa assays were performed. The results represent a mean ± S.E.

TABLE 12

COAGULATION PARAMETERS AFTER INTRAVENOUS CY 216 ADMINISTRATION IN RABBITS

Treatment (N)	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^R (seconds)
Saline Control (5)	83.2 ± 11.5	30.1 ± 3.3	24.8 ± 3.0
CY 216 1 mg/kg i.v. (5)	>150	>150	138.1 ± 7.0*
CY 216 2 mg/kg i.v. (13)	>150	>150	>296.1 ± 3.9
CY 216	>150	>150	>300

Saline or CY 216 was administered to rabbits and allowed to circulate for 10 minutes. Blood was drawn by cardiac puncture just prior to blood loss determination. APTT, Thrombin time, and Heptest^R were performed. The results represent a mean ± S.E. Statistical analysis for the heptest^R data was performed by an unpaired t test (two tailed). $t=6.332$, $df=8$, $p < .05$. * represents a significant difference from the control group.

Table 13. Saline, heparin, or CY 216 was administered to rabbits and allowed to circulate for 5 minutes at which time protamine was administered to groups which received heparin or CY 216. Blood was drawn 5 minutes after protamine administration or 10 minutes after saline administration for the control group. Blood loss was determined immediately after the blood draw. APTT, thrombin time, and Heptest^{R} were performed. The results represent the mean ± S.E. Statistical analysis was performed by a two way ANOVA.

for thrombin time
- Factor A (drug treatment):$F(2,36)=34.960$ $p < .05$
- Factor B (protamine concentration):$(2,36)=37.654$ $p < .05$
- Factor A x B:$F(4,36)=12.382$ $p < .05$

for Heptest^{R}
- Factor A (drug treatment):$F(2,36)=22.275$ $p < .05$
- Factor B (protamine concentration):$F(2,36)=19.477$ $p < .05$
- Factor A x B:$F(4,36)=5.684$ $p < .05$

Newman-Keuls multiple comparison tests were also performed.

* represents significant difference from control group.

** represents a significant difference from CY 216 (2 mg/kg i.v.) and protamine (3 mg/kg i.v.) group.

TABLE 13

COAGULATION PARAMETERS OF RABBITS ADMINISTERED INTRAVENOUS HEPARIN OR CY 216 AND PROTAMINE

Treatment (N)	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^{R} (seconds)
Saline Control (5)	83.2 ± 11.5	32.0 ± 3.1	24.8 ± 3.0
Heparin 2 mg/kg i.v. (14)	>150	>150	>300
Heparin 2 mg/kg i.v. (6) and Protamine 2 mg/kg i.v.	82.7 ± 11.5	>150	166.0 ± 55.5*
Heparin 2mg/kg i.v. (5) and Protamine 3 mg/kg i.v.	87.6 ± 7.5	33.4 ± 2.4	58.4 ± 3.7
CY 216 2 mg/kg i.v. (13)	>150	>150	>296.1 ± 3.9
CY 216 2 mg/kg i.v. (6) and Protamine 2 mg/kg i.v.	111.3 ± 24.5	73.3 ± 18.8**	138.1 ± 45.5*
CY 216 2 mg/kg i.v. (5) and Protamine 3 mg/kg i.v.	108.1 ± 14.5	47.1 ± 10.5	153 ± 47.1*

TABLE 14

COAGULATION PARAMETERS OF RABBITS ADMINISTERED SUBCUTANEOUS HEPARIN OR CY 216 AND PROTAMINE

Treatment (N)	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^R (seconds)
Saline Control (5)	83.2 ± 11.5	32.0 ± 3.1	24.8 ± 3.0
Heparin 3 mg/kg s.c. (5)	>130.8 ± 11.7	>150	>216.9 ± 49.4
Heparin 3 mg/kg s.c. (5) and Protamine 3 mg/kg i.v.	80.1 ± 9.0	27.5 ± 2.0	42.3 ± 7.3
CY 216 3 mg/kg s.c. (5)	89.2 ± 18.4	>150	67.7 ± 18.5*
CY 216 3 mg/kg s.c. (5) and Protamine 3 mg/kg i.v.	71.7 ± 16.7	31.0 ± 3.1	62.9 ± 12.5*

Saline, heparin or CY 216 was administered subcutaneously to rabbits and allowed to circulate for 125 minutes at which time protamine was administered to groups which received heparin or CY 216. Blood was drawn 5 minutes after protamine administration or 130 minutes after saline administration for the control group. Blood loss was determined immediately after blood draw. APTT, thrombin time, and Heptest^R were performed. The results represent the mean ± S.E. Statistical analysis was performed by a two way ANOVA.

for Heptest^R

Factor A (drug treatment): $F(2,17)=10.131$ $p < .05$

Factor B (protamine concentration): $F(1,17)=8.902$ $p < .05$

Factor A x B: $F(2,17)=10.365$ $p < .05$

A Newman-Keuls multiple comparison test was also performed.

* represents a significant difference from the control group.

TABLE 15

COMPARATIVE PHARMACODYNAMIC TIME COURSE
PARAMETERS OF PRIMATES ADMINISTERED HEPARIN OR CY 216

Test: Anti Xa

	Heparin (0.35 mg/kg i.v.)	CY 216 (0.35 mg/kg i.v.)
AUC (μg-hr-m^{-1})	5.97 ± 0.70	14.91 ± 2.46*
AUMC (μg-hr^2-ml^{-1})	7.83 ± 1.60	33.08 ± 7.93
MRT (min)	77.4 ± 8.68	127.80 ± 12.05*
Clp (ml-min^{-1}-kg^{-1})	1.00 ± 0.20	0.43 ± 0.05*
Vd (l-kg^{-1})	0.08 ± 0.01	0.05 ± 0.01

Primate pharmacodynamic time course parameters were determined by a noncompartmental model.

Data represents a mean ± S.E. N=4 for heparin (0.35 mg/kg i.v.) group. N=5 for CY 216 (0.35 mg/kg i.v.) group. Statistical analysis was performed by an unpaired student's t-test:

for AUC: $t=3.340$, $df=8$, $p < .05$
for MRT: $t=3.244$, $df=8$, $p < .05$
for Clp: $t=2.555$, $df=8$, $p < .05$
for Vd: $t=1.716$, $df=8$, $p > .10$ N.S.

* represents a significant difference from the heparin (0.35 mg/kg i.v.) group.

TABLE 16

COMPARATIVE PHARMACODYNAMIC TIME COURSE PARAMETERS OF PRIMATES ADMINISTERED HEPARIN OR CY 216

Test: Anti Xa

	Heparin (0.7 mg/kg i.v.)	CY 216 (0.7 mg/kg i.v.)
AUC (μg-hr-ml^{-1})	22.41 ± 3.06	19.32 ± 2.37
AUMC (μg-hr^2-ml^{-1})	21.50 ± 4.25	24.02 ± 4.78
MRT (min)	55.80 ± 5.74	71.40 ± 8.04
Clp (ml-min^{-1}-kg^{-1})	0.55 ± 0.07	0.65 ± 0.09
Vd (l-kg^{-1})	0.03 ± 0.01	0.05 ± 0.00

Primate pharmacodynamic time course parameters were determined by a noncompartmental model.

Data represents a mean ± S.E. N=5 for heparin (0.7 mg/kg i.v.) group. N=5 for CY 216 (0.7 mg/kg i.v.) group. Statistical analysis was performed by an unpaired student's t-test:

for AUC: t=0.853, df=9, p >.20 N.S.
for MRT: 5=1.549, df=9, p >.10 N.S.
for Clp: t=0.832, df=9, p >.20 N.S.
for Vd: t=1.332, df=9, p >.20 N.S.

TABLE 17

COMPARATIVE PHARMACODYNAMIC TIME COURSE PARAMETERS OF PRIMATES ADMINISTERED HEPARIN OR CY 216

Test: Anti Xa

	Heparin (0.7 mg/kg i.v.) with Protamine (0.7 mg/kg i.v.)	CY 216 (0.7 mg/kg i.v.) with Protamine (0.7 mg/kg i.v.)
AUC (μg-hr-ml^{-1})	3.73 ± 0.60	8.26 ± 1.35*
AUMC (μg-hr^2-ml^{-1})	6.09 ± 1.35	15.52 ± 3.63
MRT (min)	93.00 ± 13.90	112.70 ± 15.30
PNI	0.83 ± 0.01	0.57 ± 0.04*

Primate pharmacodynamic time course parameters were determined by a noncompartmental model.

Data represents a mean ± S.E. N=4 for heparin (0.7 mg/kg i.v.) and protamine (0.7 mg/kg i.v.) group. N=4 for CY 216 (0.7 mg/kg i.v.) and protamine (0.7 mg/kg i.v.) group. Statistical analysis was performed by an unpaired student's t-test:

for AUC: $t=3.084$, $df=7$, $p < .05$
for MRT: $t=0.862$, $df=7$, $p > .20$ N.S.
for PNI: $t=3.109$, $df=7$, $p < .05$

* represents a significant difference from the heparin (0.7 mg/kg i.v.) with protamine (0.7 mg/kg i.v.) group.

TABLE 18

COMPARATIVE PHARMACODYNAMIC TIME COURSE PARAMETERS OF PRIMATES ADMINISTERED HEPARIN OR CY 216

Test: Anti Xa

	Heparin (0.7 mg/kg i.v.) with Protamine (1.4 mg/kg i.v.)	CY 216 (0.7 mg/kg i.v.) with Protamine (1.4 mg/kg i.v.)
AUC (μg-hr-ml^{-1})	3.60 ± 0.74	9.50 ± 0.98*
AUMC (μg-hr^2-ml^{-1})	6.88 ± 2.37	19.19 ± 2.89
MRT (min)	102.60 ± 17.14	124.80 ± 20.80
PNI	0.84 ± 0.02	0.51 ± 0.06*

Primate pharmacodynamic time course parameters were determined by a noncompartmental model.

Data represents a mean ± S.E. N=5 for heparin (0.7 mg/kg i.v.) and protamine (1.4 mg/kg i.v.) group. N=4 for CY 216 (0.7 mg/kg i.v.) and protamine (1.4 mg/kg i.v.) group. Statistical analysis was performed by an unpaired student's t-test:

for AUC: $t=4.614$, $df=8$, $p < .05$
for MRT: $t=0.800$, $df=8$, $p > .20$ N.S.
for PNI: $t=5.281$, $df=8$, $p < .05$

* represents a significant difference from heparin (0.7 mg/kg i.v.) and protamine (1.4 mg/kg i.v.) group.

TABLE 19

COMPARATIVE PHARMACODYNAMIC TIME COURSE PARAMETERS OF PRIMATES ADMINISTERED HEPARIN OR CY 216

Test: Anti Xa

	Heparin (0.7 mg/kg i.v.) with Protamine (2.1 mg/kg i.v.)	CY 216 (0.7 mg/kg i.v.) with Protamine (2.1 mg/kg i.v.)
AUC ($\mu g\text{-}hr\text{-}ml^{-1}$)	2.35 ± 0.21	5.14 ± 0.64*
AUMC ($\mu g\text{-}hr^2\text{-}ml^{-1}$)	8.43 ± 3.81	5.27 ± 1.34
MRT (min)	67.80 ± 48.00	58.20 ± 7.50
PNI	0.90 ± 0.01	0.73 ± .02*

Primate pharmacodynamic time course parameters were determined by a noncompartmental model.

Data represents a mean ± S.E. N=4 for heparin (0.7 mg/kg i.v.) and protamine (2.1 mg/kg i.v.) group. N=3 for CY 216 (0.7 mg/kg i.v.) and protamine (2.1 mg/kg i.v.) group. Statistical analysis was performed by an unpaired student's t-test:

for AUC: $t=3.788$, $df=6$, $p < .05$
for MRT: $t=1.334$, $df=6$, $p > .20$ N.S.
for PNI: $t=4.362$, $df=6$, $p < .05$

* represents a significant difference from heparin (0.7 mg/kg i.v.) and protamine (2.1 mg/kg i.v.) group.

TABLE 20

COMPARATIVE PHARMACODYNAMIC TIME COURSE PARAMETERS OF PRIMATES ADMINISTERED HEPARIN OR CY 216

Test: Anti Xa

	Heparin (0.7 mg/kg s.c.)	CY 216 (0.7 mg/kg s.c.)
AUC (μg-hr-ml^{-1})	4.33 ± 1.33	31.61 ± 4.32*
AUMC (μg-hr^2-ml^{-1})	20.23 ± 6.64	178.24 ± 29.89
MRT (min)	276.00 ± 6.46	335.10 ± 11.87*
Clp (ml-min^{-1}-kg^{-1})	3.70 ± 0.44	0.39 ± 0.05*
Vd (l-kg^{-1})	0.01 ± 0.00	0.001 ± 0.00*

Primate pharmacodynamic time course parameters were determined by a noncompartmental model.

Data represents a mean ± S.E. N=5 for heparin (0.7 mg/kg s.c.) and protamine (0.7 mg/kg i.v.) group. N=4 for CY 216 (0.7 mg/kg s.c.) and protamine (0.7 mg/kg i.v.) group. Statistical analysis was performed by an unpaired student's t-test:

for AUC: $t=6.683$, $df=8$, $p < .05$
for MRT: $t=3.006$, $df=8$, $P < .05$
for Clp: $t=2.988$, $df=8$, $p < .05$
for Vd: $t=2.813$, $df=8$, $p < .05$

* represents a significant difference from heparin (0.7 mg/kg s.c.) and protamine (0.7 mg/kg i.v.) group.

TABLE 21

COMPARATIVE PHARMACODYNAMIC TIME COURSE PARAMETERS OF PRIMATES ADMINISTERED HEPARIN OR CY 216

Test: Anti Xa

	Heparin (0.7 mg/kg s.c.) with Protamine (0.7 mg/kg i.v.)	CY 216 (0.7 mg/kg s.c.) with Protamine (0.7 mg/kg i.v.)
AUC (μg-hr-ml^{-1})	2.58 ± 0.41	22.70 ± 1.40*
AUMC (μg-hr^2-ml^{-1})	13.56 ± 2.00	107.58 ± 8.05
MRT (min)	330.60 ± 37.00	286.10 ± 3.59
PNI	0.40 ± 0.09	0.20 ± 0.06

Primate pharmacodynamic time course parameters were determined by a noncompartmental model.

Data represents a mean ± S.E. N=5 for heparin (0.7 mg/kg s.c.) and protamine (0.7 mg/kg i.v.) group. N=4 for CY 216 (0.7 mg/kg s.c.) and protamine (0.7 mg/kg i.v.) group. Statistical analysis was performed by an unpaired student's t-test:

for AUC: $t=13.843$, $df=8$, $p < .05$
for MRT: $t=1.058$, $df=8$, $p > .20$ N.S.
for PNI: $t=1.655$, $df=8$, $p > .10$ N.S.

* represents a significant difference from heparin (0.7 mg/kg s.c.) with protamine (0.7 mg/kg i.v.) group.

TABLE 22

COMPARATIVE PHARMACODYNAMIC TIME COURSE PARAMETERS OF PRIMATES ADMINISTERED HEPARIN OR CY 216

Test: Anti-Xa

	CY 216 (0.7 mg/kg s.c.) with Protamine (1.4 mg/kg i.v.)
AUC ($\mu g\text{-}ml^{-1}$)	22.30 ± 1.30
AUMC ($\mu g\text{-}hr^{2}\text{-}ml^{-1}$)	89.34 ± 17.77
MRT (min)	237.00 ± 35.1
PNI	0.21 ± 0.05

Primate pharmacodynamic time course parameters were determined by a noncompartmental model.

Data represents a mean ± S.E. N=5 for CY 216 (0.7 mg/kg s.c.) and protamine (1.4 mg/kg i.v.) group.

TABLE 23

COMPARATIVE PHARMACODYNAMIC TIME COURSE PARAMETERS OF PRIMATES ADMINISTERED HEPARIN OR CY 216

Test: Heptest^{R}

	Heparin (0.35 mg/kg i.v.)	CY 216 (0.35 mg/kg) i.v.)
AUC (μg-hr-ml^{-1})	3.73 ± 0.56	6.54 ± 1.67*
AUMC (μg-hr^{2}-ml^{-1})	1.55 ± 1.00	11.20 ± 0.45
MRT (min)	24.00 ± 12.00	114.72 ± 13.68*
Clp (ml-min^{-1}-kg^{-1})	1.59 ± 0.23	1.07 ± 0.18*
Vd (l-kg^{-1})	0.04 ± 0.02	0.02 ± 0.01

Primate pharmacodynamic time course parameters were determined by a noncompartmental model.

Data represents a mean ± S.E. N=3 for heparin (0.35 mg/kg i.v.) group. N=4 for CY 216 (0.35 mg/kg i.v.) group. Statistical analysis was performed by an unpaired student's t-test:

for AUC: $t=3.476$, $df=6$, $p < .05$
for MRT: $t=4.128$, $df=6$, $p < .05$
for Clp: $t=6.666$, $df=6$, $p < .05$
for Vd: $t=0.398$, $df=6$, $p > .70$ N.S.

* represents a significant difference from heparin (0.35 mg/kg i.v.) group.

TABLE 24

COMPARATIVE PHARMACODYNAMIC TIME COURSE PARAMETERS OF PRIMATES ADMINISTERED HEPARIN OR CY 216

Test: Heptest^{R}

	Heparin (0.7 mg/kg i.v.)	CY 216 (0.7 mg/kg) i.v.)
AUC (μg-hr-ml^{-1})	18.36 ± 1.28	27.15 ± 3.45*
AUMC (μg-hr^{2}-ml^{-1})	18.37 ± 2.94	24.84 ± 5.56
MRT (min)	58.80 ± 6.79	53.28 ± 7.27
Clp (ml-min^{-1}-kg^{-1})	0.65 ± 0.05	0.56 ± 0.14*
Vd (l-kg^{-1})	0.04 ± 0.01	0.02 ± 0.00

Primate pharmacodynamic time course parameters were determined by a noncompartmental model.

Data represents a mean ± S.E. N=5 for both groups. Statistical analysis was performedby an unpaired student's t-test:

for AUC: t=2.540, df=9, $p < .05$
for MRT: t=0.128, df=9, $p > .50$ N.S.
for Clp: t=2.488, df=9, $p < .05$
for Vd: t=0.416, df=9, $p > .50$ N.S.

* represents a significant difference from the heparin (0.7 mg/kg i.v.) group.

TABLE 25

COMPARATIVE PHARMACODYNAMIC TIME COURSE PARAMETERS OF PRIMATES ADMINISTERED HEPARIN OR CY 216

Test: Heptest[R]

	Heparin (0.7 mg/kg i.v.) with Protamine (0.7 mg/kg i.v.)	CY 216 (0.7 mg/kg i.v.) with Protamine (0.7 mg/kg i.v.)
AUC (μg-hr-ml^{-1})	12.41 ± 2.14	18.96 ± 0.64*
AUMC (μg-hr^2-ml^{-1})	12.63 ± 2.99	23.90 ± 3.31
MRT (min)	59.40 ± 7.50	75.20 ± 9.01
PNI	0.30 ± 0.06	0.30 ± 0.01

Primate pharmacodynamic time course parameters were determined by a noncompartmental model.

Data represents a mean ± S.E. N=4 for both groups. Statistical analysis was performed by an unpaired student's t-test:

for AUC: t=2.601, df=7, p >.05
for MRT: t=1.187, df=7, p >.20 N.S.
for PNI: t=0.186, df=7, p >.50 N.S.

* represents a significant difference from the heparin (0.7 mg/kg i.v.) with protamine (0.7 mg/kg i.v.) group.

TABLE 26

COMPARATIVE PHARMACODYNAMIC TIME COURSE PARAMETERS OF PRIMATES ADMINISTERED HEPARIN OR CY 216

Test: Heptest[R]

	Heparin (0.7 mg/kg i.v.) with Protamine (1.4 mg/kg i.v.)	CY 216 (0.7 mg/kg i.v.) with Protamine (1.4 mg/kg i.v.)
AUC (μg-hr-ml^{-1})	3.72 ± 0.52	12.01 ± 1.09*
AUMC (μg-hr^2-ml^{-1})	5.42 ± 1.48	9.13 ± 1.70
MRT (min)	81.60 ± 10.98	46.20 ± 4.20*
PNI	0.80 ± 0.01	0.56 ± 0.04*

Primate pharmacodynamic time course parameters were determined by a noncompartmental model.

Data represents a mean ± S.E. N=5 for heparin (0.7 mg/kg i.v.) and protamine (1.4 mg/kg i.v.) group. N=4 for CY 216 (0.7 mg/kg i.v.) and protamine (1.4 mg/kg i.v.) group. Statistical analysis was performed by an unpaired student's t-test:

for AUC: $t=3.878$, $df=8$, $p < .05$
for MRT: $t=9.043$, $df=8$, $p < .05$
for PNI: $t=4.247$, $df=8$, $p > .05$

* represents a significant difference from heparin (0.7 mg/kg iv.) and protamine (1.4 mg/kg i.v.) group.

TABLE 27

COMPARATIVE PHARMACODYNAMIC TIME COURSE PARAMETERS OF PRIMATES ADMINISTERED HEPARIN OR CY 216

Test: Heptest^{R}

	Heparin (0.7 mg/kg i.v.) with Protamine (2.1 mg/kg i.v.)	CY 216 (0.7 mg/kg i.v.) with Protamine (2.1 mg/kg i.v.)
AUC (μg-hr-ml^{-1})	2.17 ± 0.27	11.210 ± 0.64*
AUMC (μg-hr^{2}-ml^{-1})	2.73 ± 1.13	19.250 ± 4.94
MRT (min)	75.20 ± 28.40	100.200 ± 21.70*
PNI	0.88 ± 0.01	0.59 ± 0.01

Primate pharmacodynamic time course parameters were determined by a noncompartmental model.

Data represents a mean ± S.E. N=4 for heparin (0.7 mg/kg i.v.) and protamine (2.1 mg/kg i.v.) group. N=3 for CY 216 (0.7 mg/kg i.v.) and protamine (2.1 mg/kg i.v.) group. Statistical anqalysis was performed by an unpaired student's t-test:

for AUC: $t=12.862$, $df=6$, $p < .05$
for MRT: $t=1.755$, $df=6$, $p < .10$ N.S.
for PNI: $t=11.218$, $df=6$, $p < .05$

* represents a significant difference from heparin (0.7 mg/kg i.v.) and protamine (2.1 mg/kg i.v.) group.

TABLE 28

COMPARATIVE PHARMACODYNAMIC TIME COURSE PARAMETERS OF PRIMATES ADMINISTERED HEPARIN OR CY 216

Test: Heptest

	Heparin (0.7 mg/kg s.c.)	CY 216 (0.7 mg/kg) s.c.)
AUC (μg-hr-ml^{-1})	4.33 ± 1.33	31.61 ± 4.32*
AUMC (μg-hr^2-ml^{-1})	20.23 ± 6.64	178.24 ± 29.89
MRT (min)	276.00 ± 6.46	335.10 ± 11.87*
Clp (ml-min^{-1}-kg^{-1})	3.70 ± 0.44	0.39 ± 0.05*
Vd (l-kg^{-1})	0.01 ± 0.00	0.001 ± 0.00*

Primate pharmacodynamic time course parameters were determined by a noncompartmental model.

Data represents a mean ± S.E. N=5 for heparin (0.7 mg/kg s.c.) and protamine (0.7 mg/kg i.v.) group. N=4 for CY 216 (0.7 mg/kg s.c.) and protamine (0.7 mg/kg i.v.) group. Statistical analysis was performed by an unpaired student's t-test:

for AUC: $t=6.683$, $df=8$, $p < .05$
for MRT: $t=3.006$, $df=8$, $p < .05$
for Clp: $t=2.988$, $df=8$, $p < .05$
for Vd: $t=2.813$, $df=8$, $p < .05$

* represents a significant difference from the heparin (0.7 mg/kg s.c.) group.

TABLE 29

COMPARATIVE PHARMACODYNAMIC TIME COURSE PARAMETERS OF PRIMATES ADMINISTERED HEPARIN OR CY 216

Test: Heptest^{R}

	Heparin (0.7 mg/kg s.c.) with Protamine (0.7 mg/kg i.v.)	CY 216 (0.7 mg/kg s.c.) with Protamine (0.7 mg/kg i.v.)
AUC (μg-hr-ml^{-1})	2.77 ± 0.29	20.62 ± 3.86*
AUMC (μg-hr^{2}-ml^{-1})	8.93 ± 1.08	77.86 ± 16.28
MRT (min)	192.60 ± 10.45	225.00 ± 7.17*
PNI	0.51 ± 0.05	0 ± 0.19*

Primate pharmacodynamic time course parameters were determined by a noncompartmental model.

Data represents a mean ± S.E. N=5 for heparin (0.7 mg/kg s.c.) and protamine (0.7 mg/kg i.v.) group. N=4 for CY 216 (0.7 mg/kg s.c.) and protamine (0.7 mg/kg i.v.) group. Statistical analysis was performed by an unpaired student's t-test:

for AUC: $t=5.110$, $df=8$, $p < .05$
for MRT: $t=2.401$, $df=8$, $P < .05$
for PNI: $t=8.746$, $df=8$, $p > .05$

* represents a significant difference from heparin (0.7 mg/kg s.c.) and protamine (0.7 mg/kg i.v.) group.

TABLE 30

COMPARATIVE PHARMACODYNAMIC TIME COURSE PARAMETERS OF PRIMATES ADMINISTERED HEPARIN OR CY 216

Test: Heptest^{R}

	CY 216 (0.7 mg/kg s.c.) with Protamine (1.4 mg/kg i.v.)
AUC ($\mu g\text{-}ml^{-1}$)	10.08 ± 1.47
AUMC ($\mu g\text{-}hr^{2}\text{-}ml^{-1}$)	42.36 ± 4.44
MRT (min)	257.40 ± 11.70
PNI	0.03 ± 0.14

Primate pharmacdynamic time course parameters were determined by a noncompartmental model.

Data represents a mean ± S.E. N=5 for CY 216 (0.7 mg/kg s.c.) and protamine (1.4 mg/kg i.v.) group.

TABLE 31

% NEUTRALIZATION OF INTRAVENOUSLY ADMINISTERED HEPARIN OR CY 216

Test: Anti-Xa

Protamine:Heparin (mg/kg) or Protamine:CY 216 (mg/kg)	1:1		2:1		3:1	
	%N	(% I.)*	%N	(% I.)*	%N	(% I.)*
Heparin (100 μg/kg i.v.) in rabbits	90	(25.0)	90	(25.0)	-	-
Heparin (2 mg/kg i.v.) in rabbits	96	(70.2)	100	(70.2)	-	-
Heparin (0.7 mg/kg i.v.) in primates	88	(92)	89.6	(92.0)	100	(92)
CY 216 (100 μg/kg i.v.) in rabbits	0	(1.8)	0	(1.8)	-	-
CY 216 (2 mg/kg i.v.) in rabbits	50	(50.9)	75	(50.9)	-	-
CY 216 (0.7 mg/kg i.v.) in primates	89.3	(91)	79.8	(91)	92.7	(91)

* = heparin or CY 216 activity (pre protamine).

Percent neutralization for the amidolytic anti Xa data obtained from the rabbit stasis thrombosis experiments (100 μg/kg i.v.), the rabbit blood loss experiments (2 mg/kg i.v.) and the primate pharmacodynamic experiments were calculated as described in methods. Percent N was calculated at the 30 minute time period for the primates and the 10 minute time period for the rabbits.

TABLE 32

% NEUTRALIZATION OF INTRAVENOUSLY ADMINISTERED HEPARIN OR CY 216

Test: Anti-IIa

Protamine:Heparin (mg/kg) or Protamine:CY 216 (mg/kg)	1:1		2:1		3:1	
	%N	(% I.)*	%N	(% I.)*	%N	(% I.)*
Heparin (100 μg/kg i.v.) in rabbits	96	(32.4)	92	(32.4)	-	-
Heparin (2 mg/kg i.v.) in rabbits	94	(92.4)	99	(92.4)	-	-
Heparin (0.7 mg/kg i.v.) in primates	91	(90.6)	89.6	(90.6)	100	(90.6)
CY 216 (100 μg/kg i.v.) in rabbits	0	(5.8)	0	(5.8)	-	-
CY 216 (2 mg/kg i.v.) in rabbits	64	(72.1)	100	(72.1)	-	-
CY 216 (0.7 mg/kg i.v.) in primates	89	(68.2)	80	(68.2)	83	(68.2)

* = heparin or CY 216 activity (pre protamine).

Percent neutralization for the amidolytic anti IIa data obtained from the rabbit stasis thrombosis experiments (100 μg/kg i.v.), the rabbit blood loss experiments (2 mg/kg i.v.) and the primate pharmacodynamic experiments were calculated as described in methods. Percent N was calculated at the 30 minute time period for the primates and the 10 minute time period for the rabbits.

TABLE 33

% NEUTRALIZATION OF SUBCUTANEOUSLY ADMINISTERED HEPARIN OR CY 216

Test: Anti-Xa

Protamine:Heparin (mg/kg) or Protamine:CY 216 (mg/kg) 1:1	% N	(% I.)*
Heparin (1 mg/kg s.c.) in rabbits	19	(15.8)
Heparin (2.5 mg/kg s.c.) in rabbits	94	(30.0)
Heparin (0.7 mg/kg s.c.) in primates	18	(7.3)
CY 216 (1 mg/kg s.c.) in rabbits	33	(22.5)
CY 216 (2.5 mg/kg s.c.) in rabbits	31	(40.3)
CY 216 (0.7 mg/kg s.c.) in primates	41	(53.0)

* = heparin or CY 216 activity (pre protamine).

Percent neutralization for the amidolytic anti Xa data obtained from the rabbit stasis thrombosis experiments (1 mg/kg s.c.), the rabbit ear blood loss experiments (2.5 mg/kg s.c.) and the primate pharmacodynamic experiments was calculated as described in methods. Percent N was calculated at the 245 minute time period for the primates and the 120 time period for the rabbits.

TABLE 34

% NEUTRALIZATION OF SUBCUTANEOUSLY ADMINISTERED HEPARIN OR CY 216

Test: Anti-IIa

Protamine:Heparin (mg/kg) or Protamine:CY 216 (mg/kg) 1:1	% N	(% I.)*
Heparin (1 mg/kg s.c.) in rabbits	14	(16.2)
Heparin (2.5 mg/kg s.c.) in rabbits	95	(73.0)
Heparin (0.7 mg/kg s.c.) in primates	28.6	(7.0)
CY 216 (1 mg/kg s.c.) in rabbits	0	(7.0)
CY 216 (2.5 mg/kg s.c.) in rabbits	91	(51.3)
CY 216 (0.7 mg/kg s.c.) in primates	71	(13.0)

* = heparin or CY 216 activity (pre protamine).

Percent neutralization for the amidolytic anti IIa data obtained from the rabbit stasis thrombosis experiments (1 mg/kg s.c.), the rabbit ear blood loss experiments (2.5 mg/kg s.c.) and the primate pharmacodynamic experiments was calculated as described in methods. Percent N was calculated at the 245 minute time period for the primates and the 120 time period for the rabbits.

FIGURES

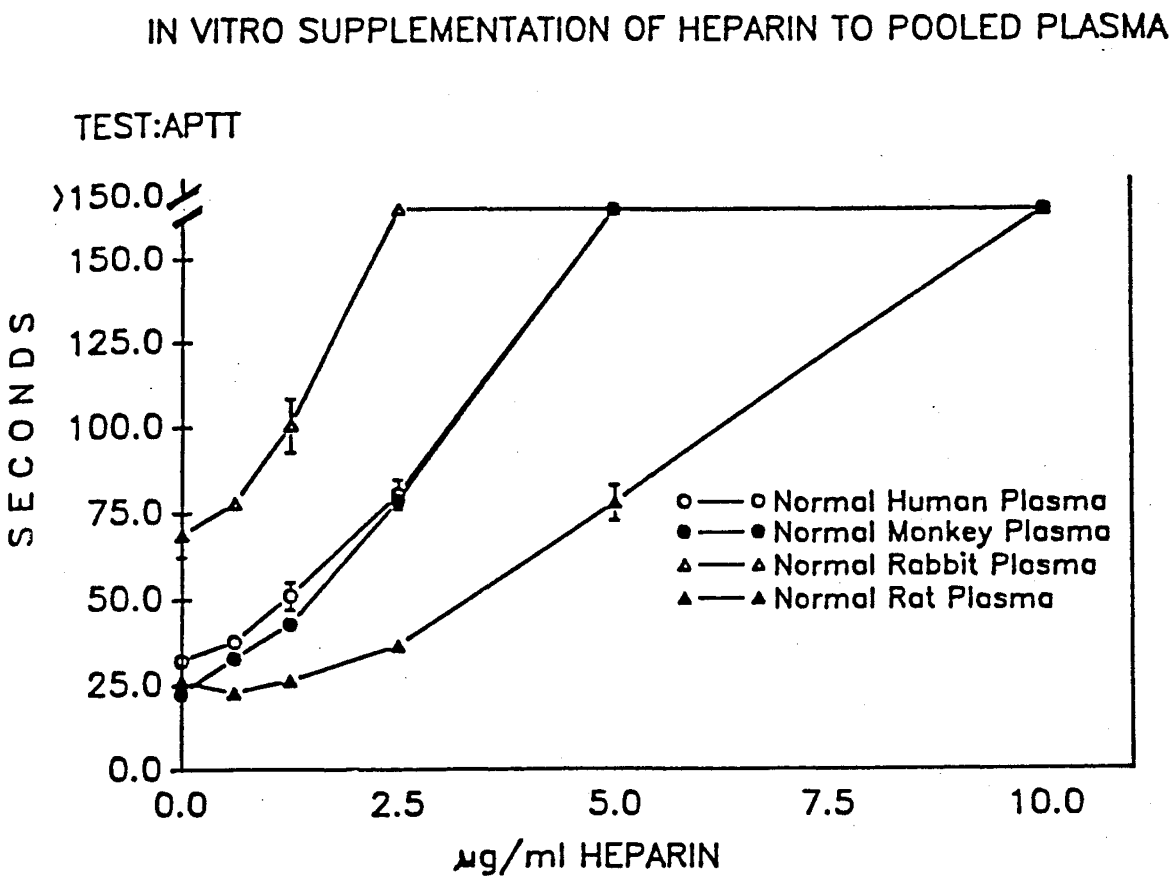


Figure 1. Heparin was supplemented to NHP, NMP, NRP and NratP in a concentration range of 0-10 µg/ml. Anticoagulant activity was determined by the APTT. Data represents a mean ± S.E. The experiment was performed in triplicate on seperate days.

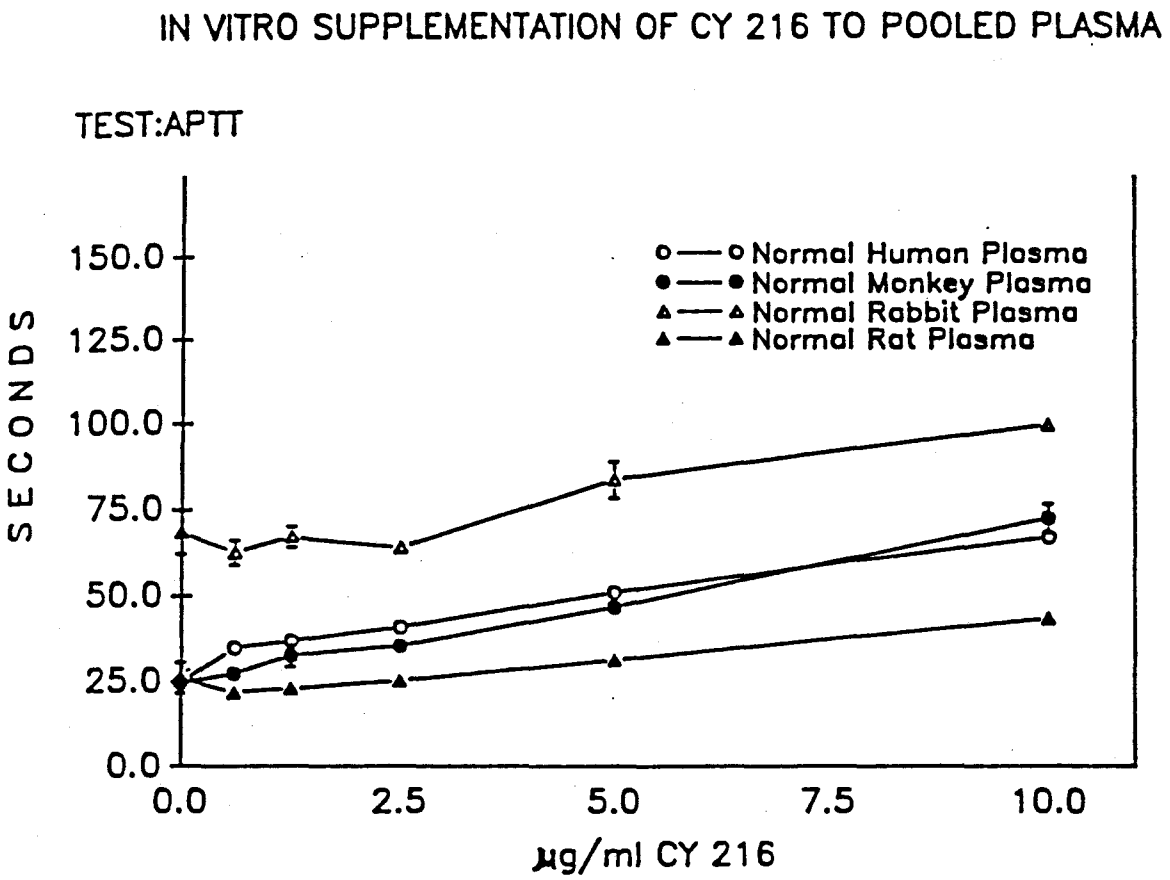


Figure 2. CY 216 was supplemented to NHP, NMP, NRP and NratP in a concentration range of 0-10 µg/ml. Anticoagulant activity was determined by the APTT. Data represents a mean ± S.E. The experiment was performed in triplicate on separate days.

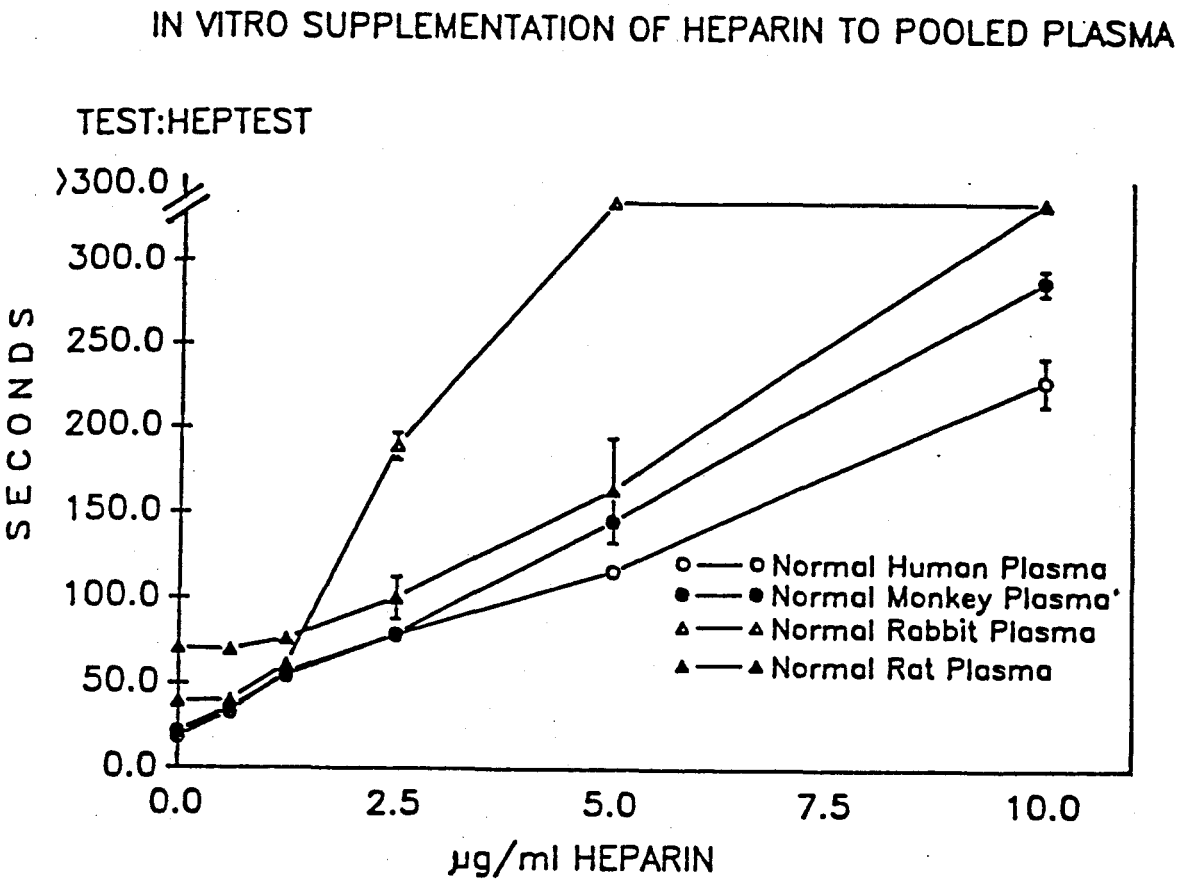


Figure 3. Heparin was supplemented to NHP, NMP, NRP and NratP in a concentration range of 0-10 µg/ml. Antiprotease activity was determined by the Heptest®. Data represents a mean ± S.E. The experiment was performed in triplicate on separate days.

IN VITRO SUPPLEMENTATION OF CY 216 TO POOLED PLASMA

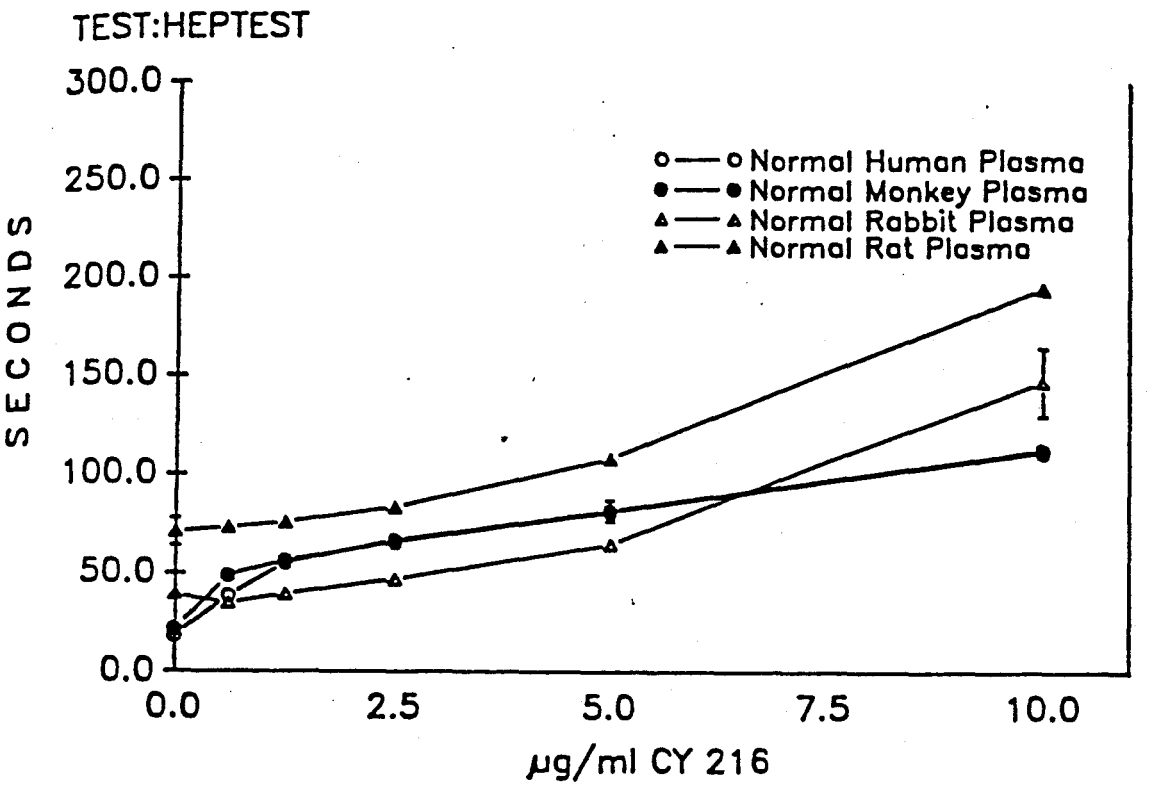


Figure 4. CY 216 was supplemented to NHP, NMP, NRP and NratP in a concentration range of 0-10 µg/ml. Antiprotease activity was determined by the Heptest®. Data represents a mean ± S.E. The experiment was performed in triplicate on separate days.

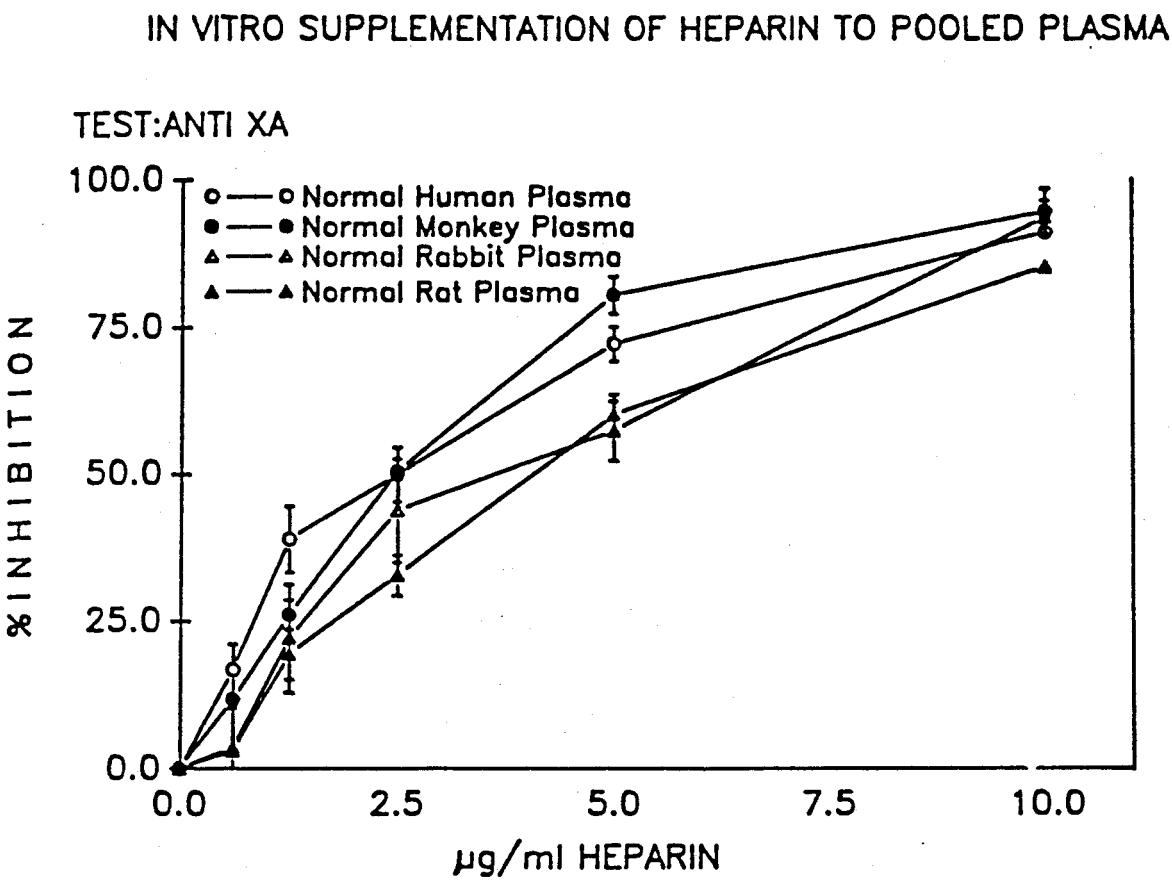


Figure 5. Heparin was supplemented to NHP, NMP, NRP and NratP in a concentration range of 0-10 µg/ml. Antiprotease activity was determined by an amidolytic anti Xa assay. Data represents a mean ± S.E. The experiment was performed in triplicate on separate days.

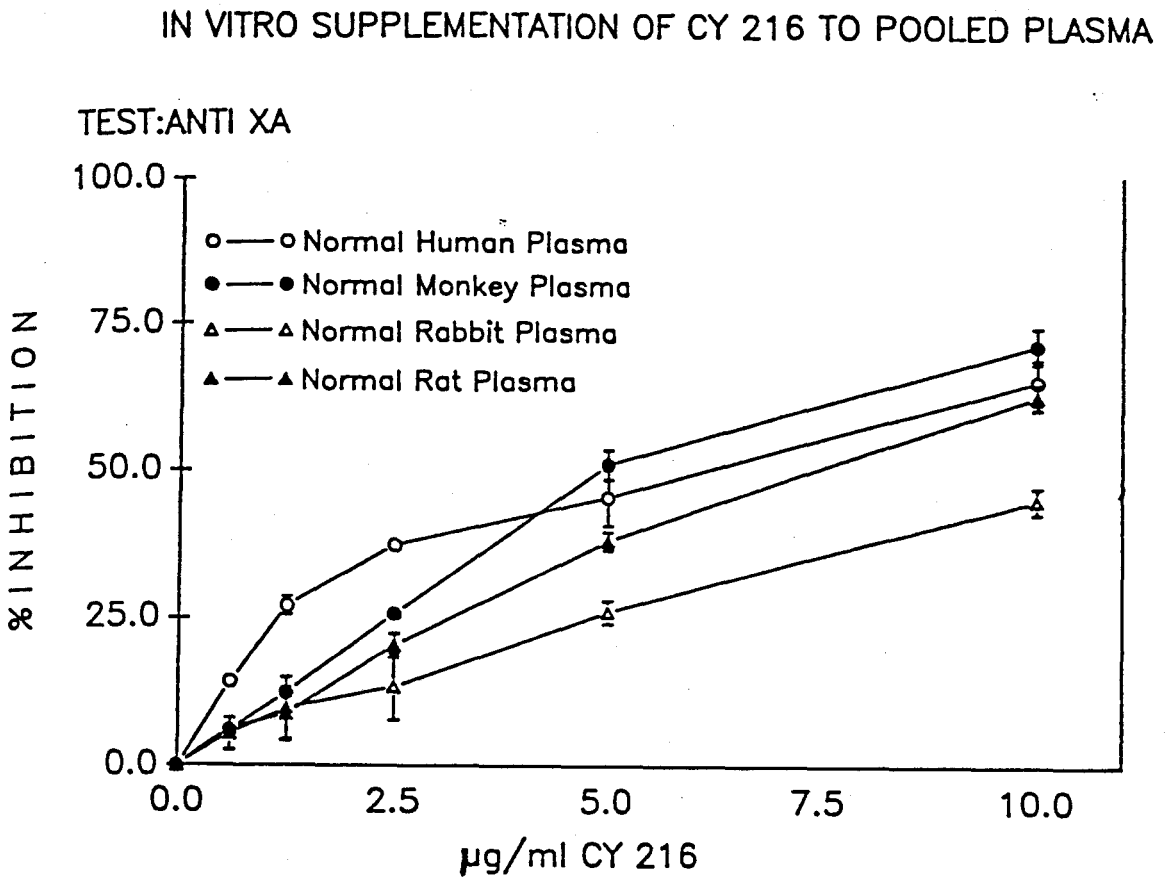


Figure 6. CY 216 was supplemented to NHP, NMP, NRP and NratP in a concentration range of 0-10 µg/ml. Antiprotease activity was determined by an amidolytic anti Xa assay. Data represents a mean ± S.E. The experiment was performed in triplicate on separate days.

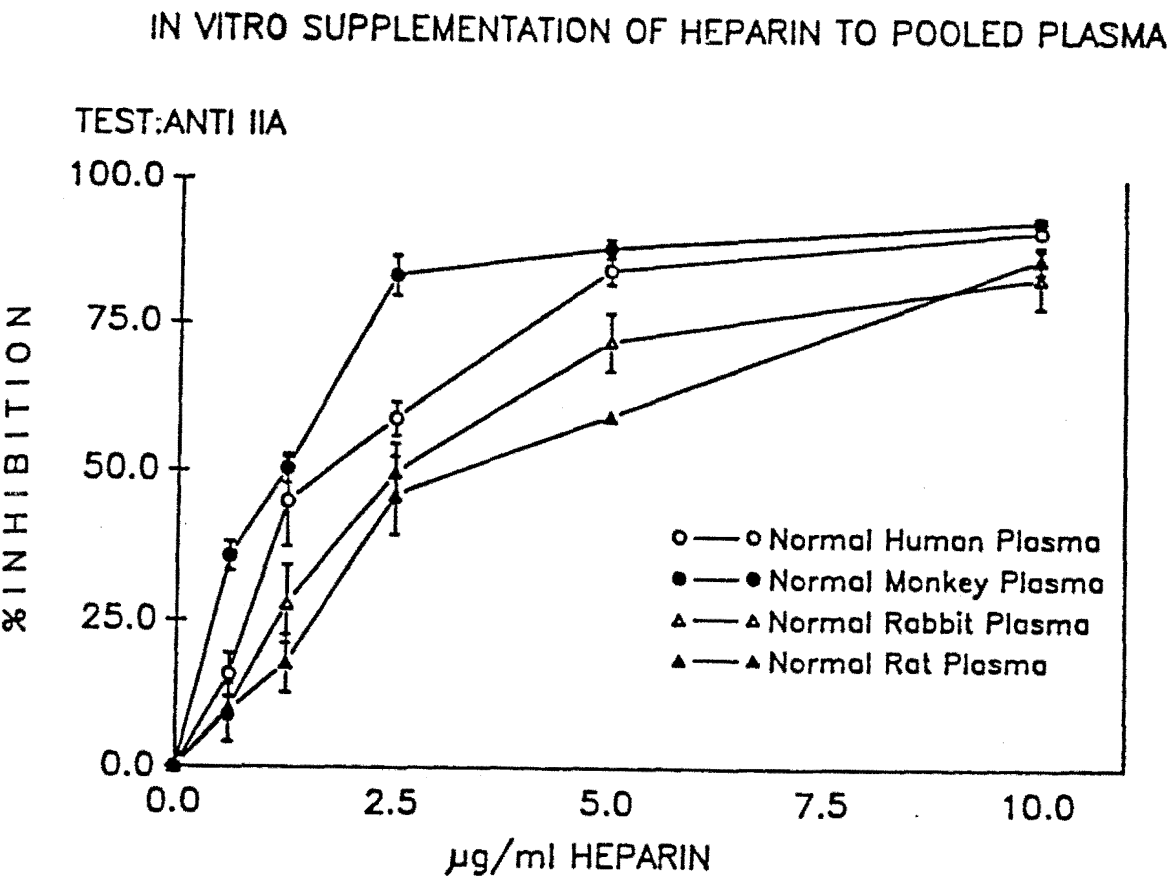


Figure 7. Heparin was supplemented to NHP, NMP, NRP and NratP in a concentration range of 0-10 µg/ml. Antiprotease activity was determined by an amidolytic anti IIa assay. Data represents a mean ± S.E. The experiment was performed in triplicate on separate days.

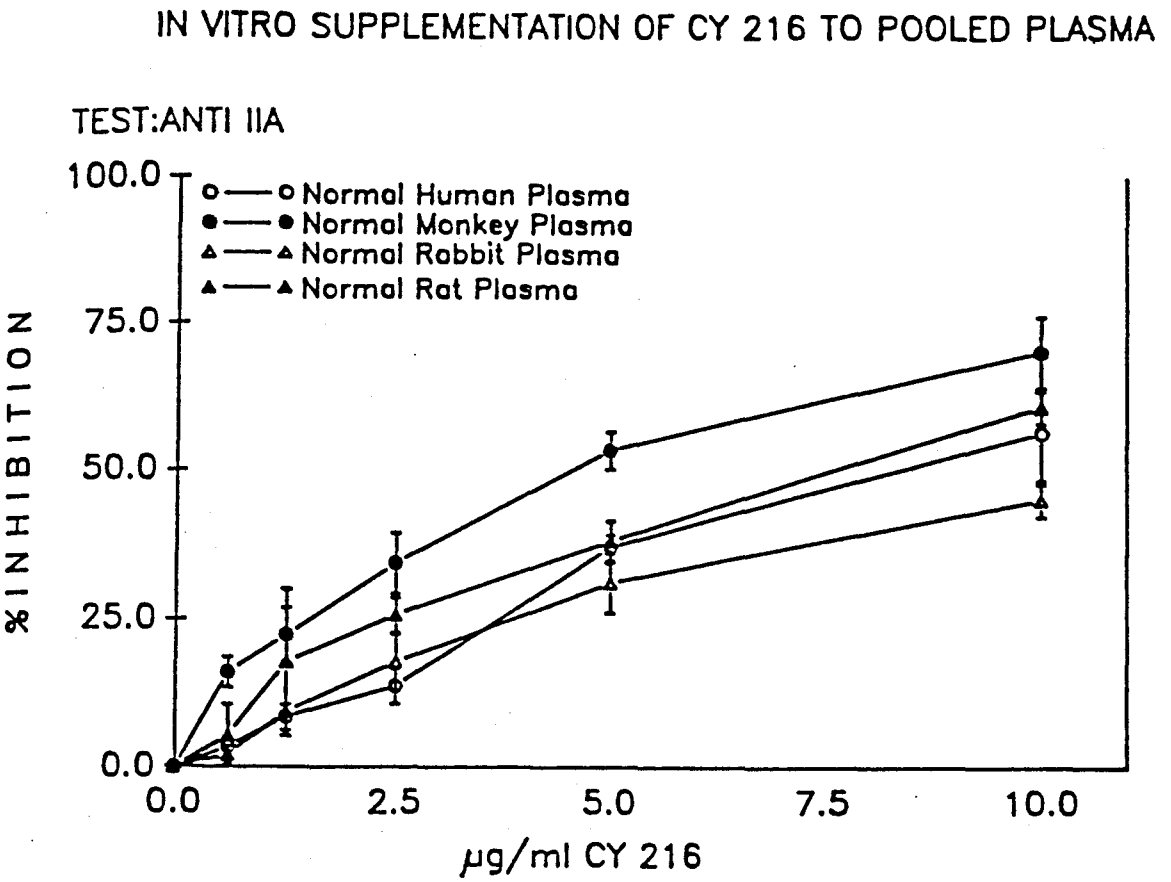
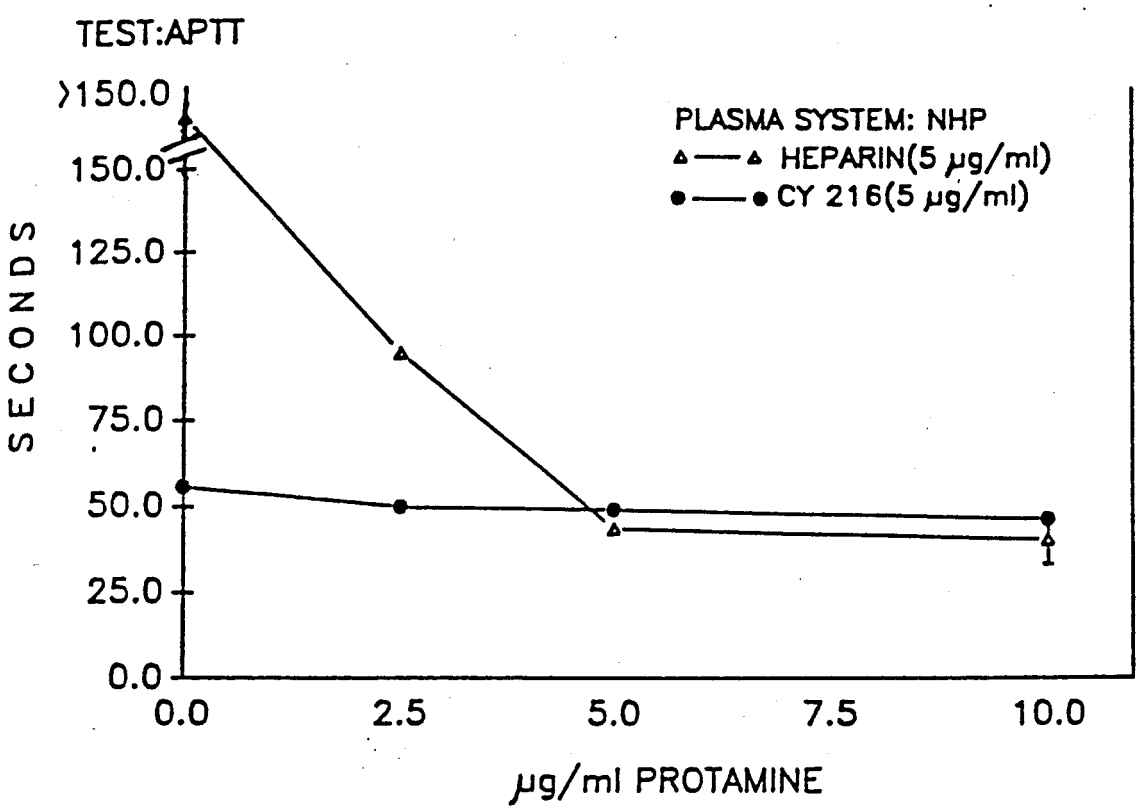


Figure 8. CY 216 was supplemented to NHP, NMP, NRP and NratP in a concentration range of 0-10 µg/ml. Antiprotease activity was determined by an amidolytic anti IIa assay. Data represents a mean ± S.E. The experiment was performed in triplicate on separate days.

Figure 9. Heparin or CY 216 was supplemented to NHP (upper panel) or to NMP (lower panel) to obtain a final concentration of 5 μg/ml. Protamine sulfate was added to the heparin and CY 216 solutions to obtain final protamine concentrations of 2.5 μg/ml, 5.0 μg/ml and 10 μg/ml. Anticoagulant activity was determined using the APTT. The control value for NHP was 33.2 ± 0.4 seconds and the control value for NMP was 22.3 ± 0.3 seconds. Data represents a mean ± S.E. The experiments were performed five times on separate days.

IN VITRO ANTICOAGULANT ACTIVITY OF HEPARIN AND CY 216
AFTER PROTAMINE ADMINISTRATION



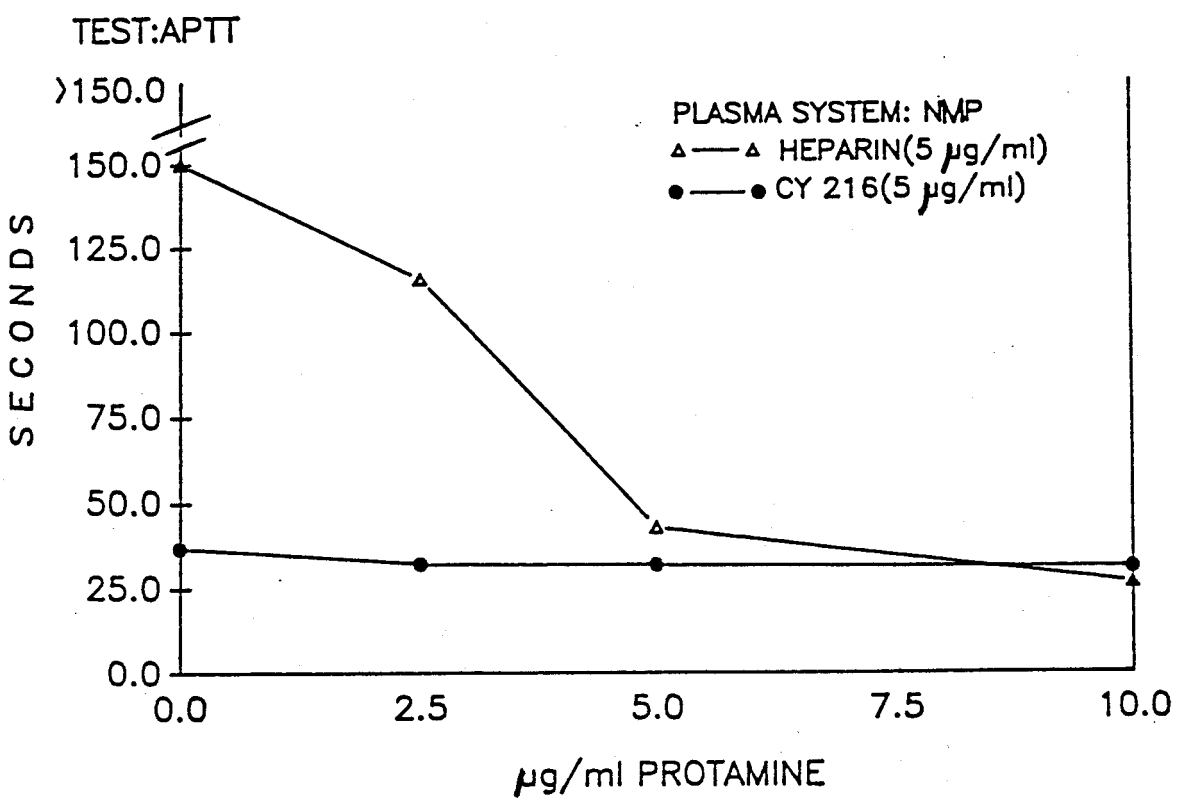
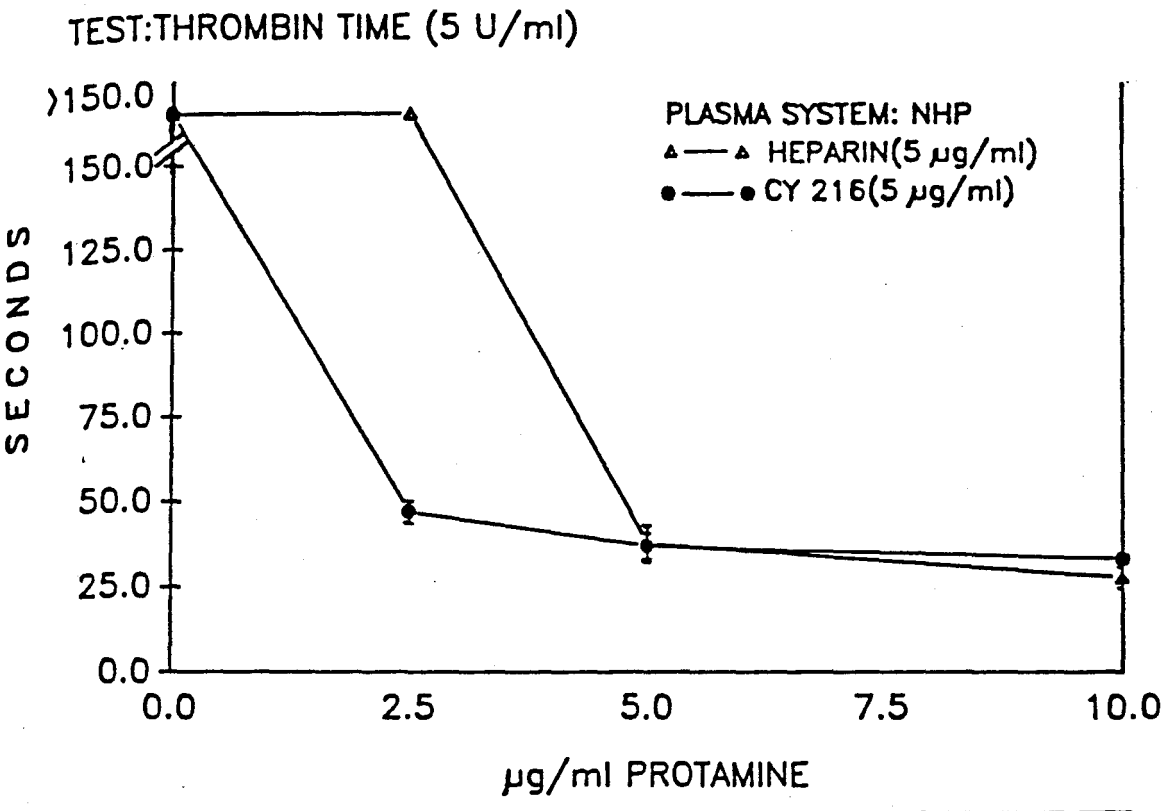


Figure 10. Heparin or CY 216 was supplemented to NHP (upper panel) or to NMP (lower panel) to obtain a final concentration of 5 μg/ml. Protamine sulfate was added to the heparin and CY 216 solutions to obtain final protamine concentrations of 2.5 μg/ml, 5.0 μg/ml and 10 μg/ml. Anticoagulant activity was determined using the thrombin time. The control value for NHP was 27.2 ± 0.7 seconds and the control value for NMP was 33.5 ± 0.3 seconds. Data represents a mean ± S.E. The experiments were performed five times on separate days.

IN VITRO ANTICOAGULANT ACTIVITY OF HEPARIN AND CY 216 AFTER PROTAMINE ADMINISTRATION



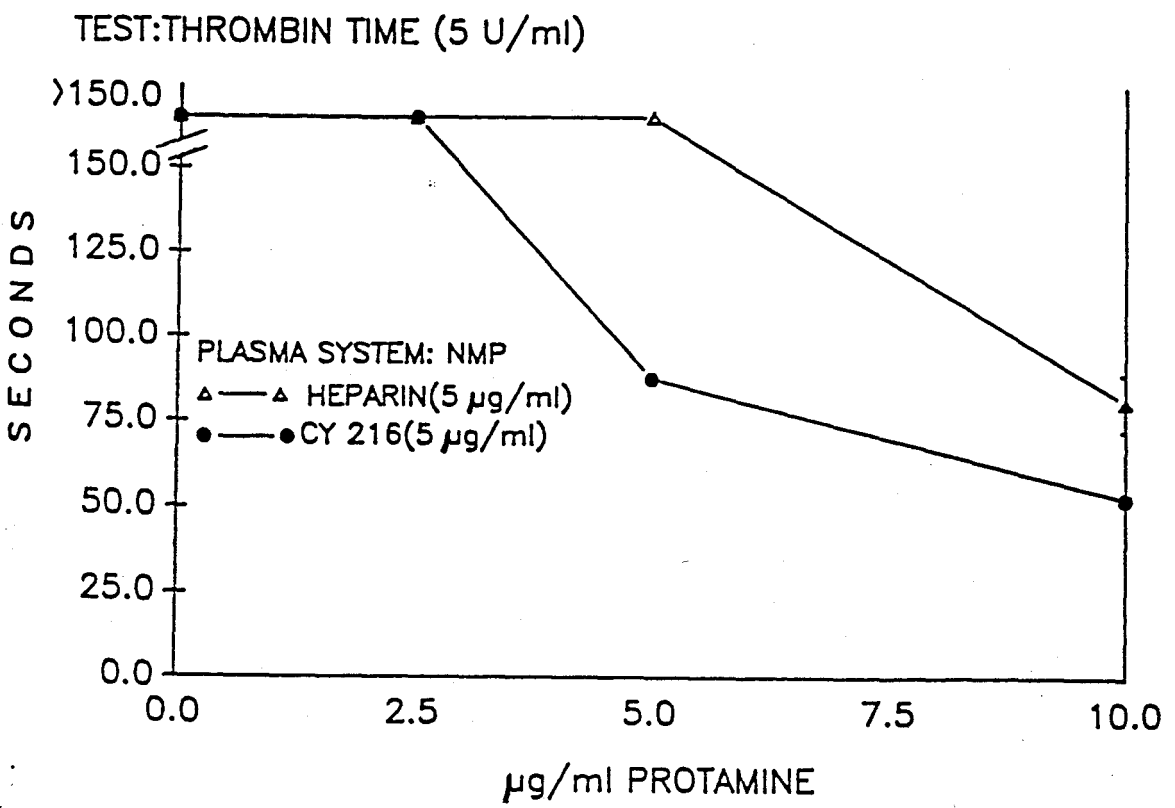
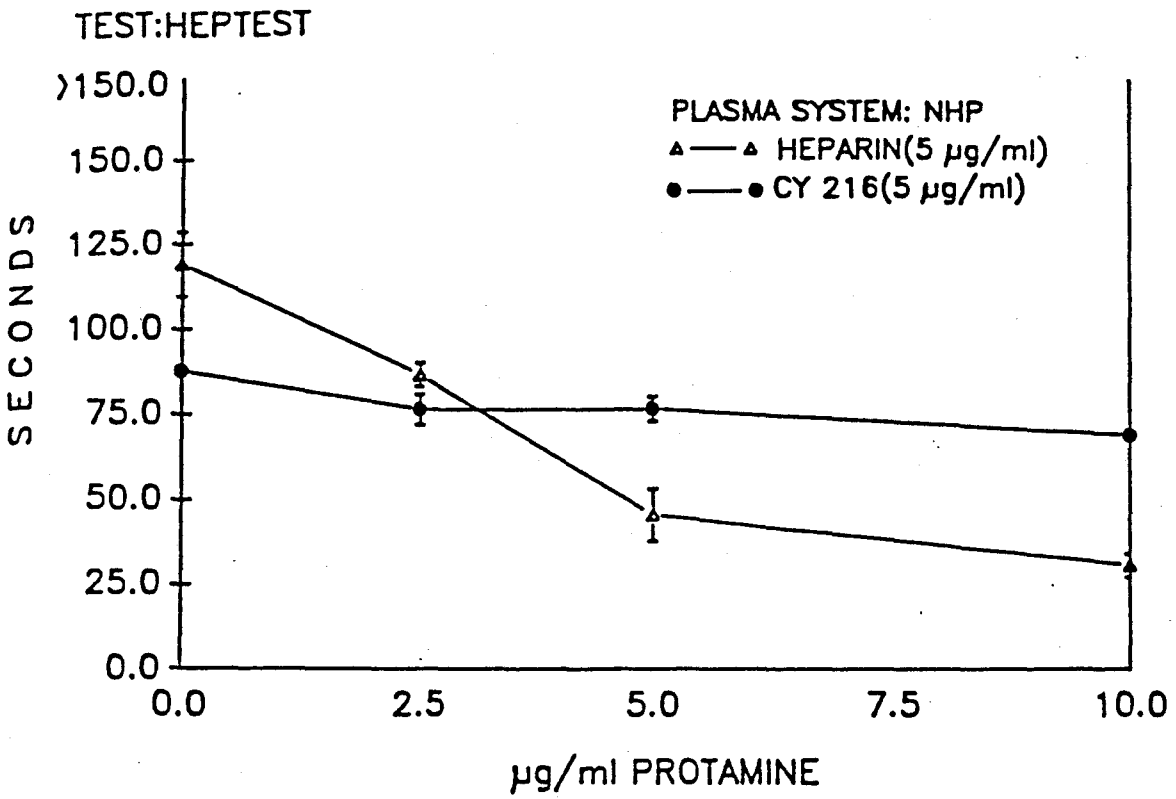


Figure 11. Heparin or CY 216 was supplemented to NHP (upper panel) or to NMP (lower panel) to obtain a final concentration of 5 μg/ml. Protamine sulfate was added to the heparin and CY 216 solutions to obtain final protamine concentrations of 2.5 μg/ml, 5.0 μg/ml and 10 μg/ml. Anticoagulant activity was determined using the Heptest®. The control value for NHP was 17.6 ± 1.6 seconds and the control value for NMP was 21.5 ± 0.1 seconds. Data represents a mean ± S.E. The experiments were performed five times on separate days.

IN VITRO ANTICOAGULANT ACTIVITY OF HEPARIN AND CY 216
AFTER PROTAMINE ADMINISTRATION



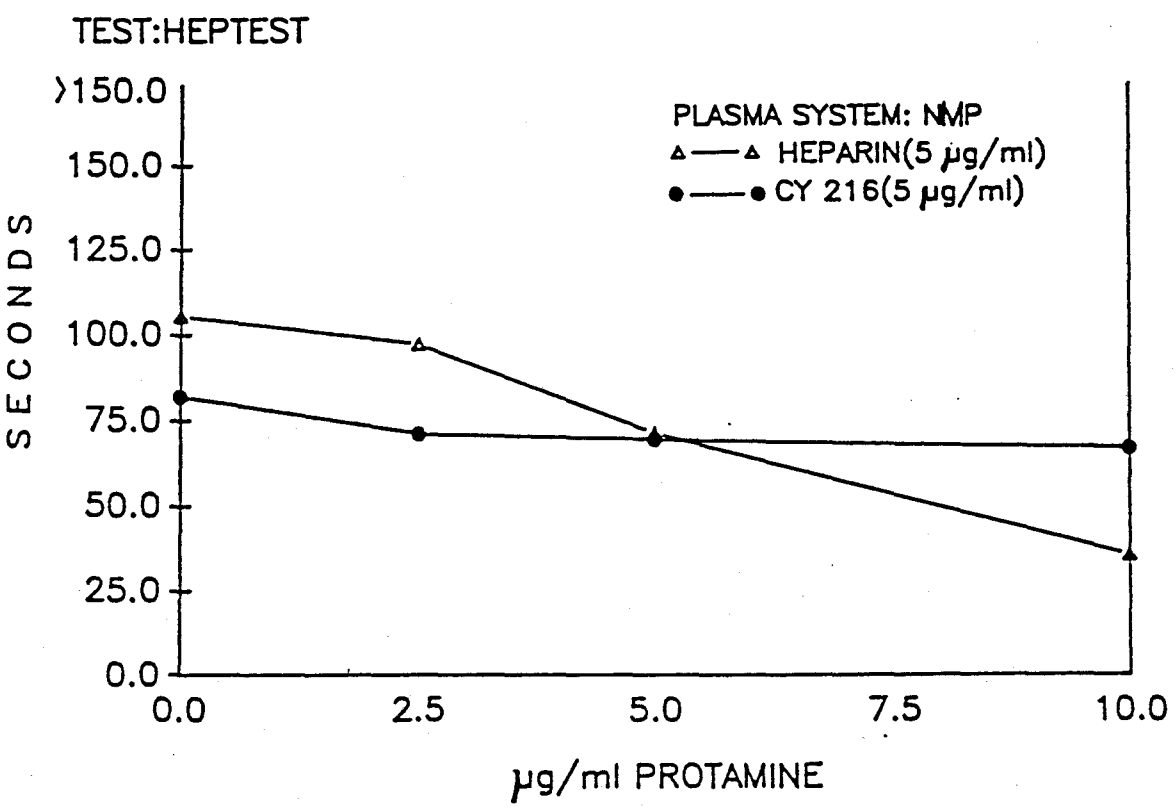
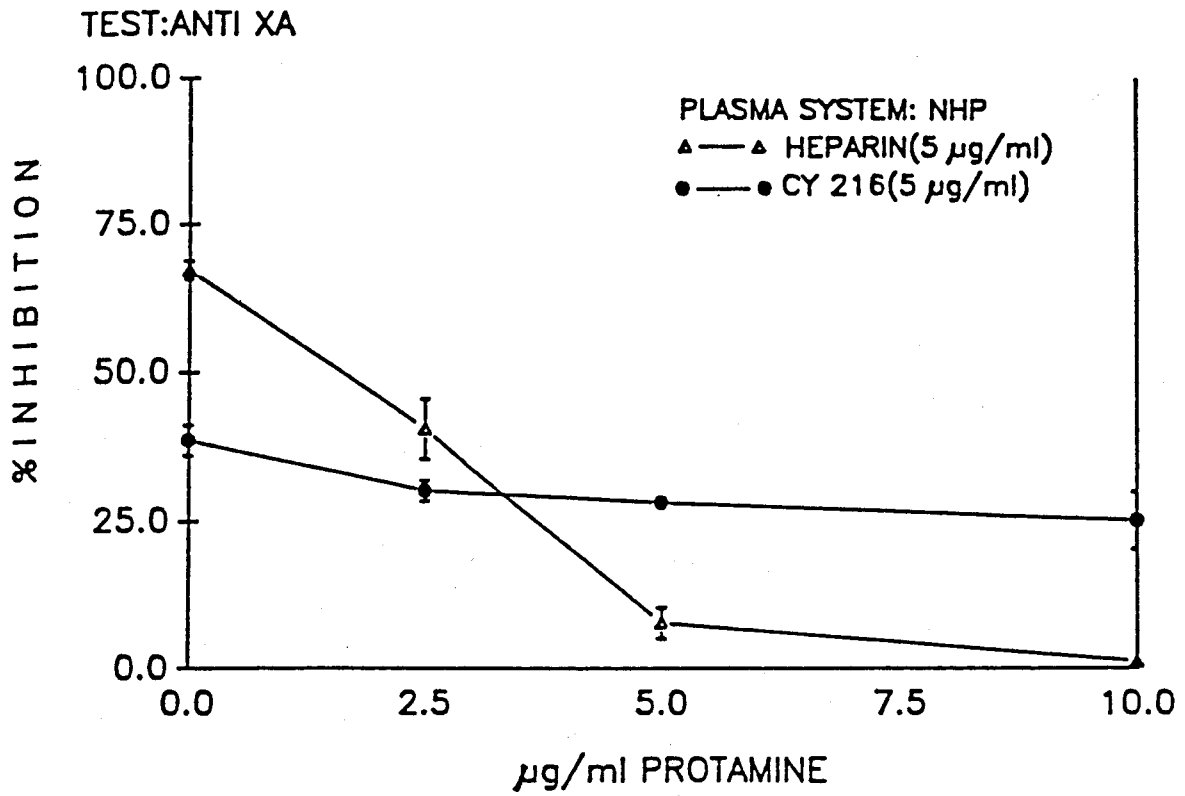


Figure 12. Heparin or CY 216 was supplemented to NHP (upper panel) or to NMP (lower panel) to obtain a final concentration of 5 μg/ml. Protamine sulfate was added to the heparin and CY 216 solutions to obtain final protamine concentrations of 2.5 μg/ml, 5.0 μg/ml and 10 μg/ml. Antiprotease activity was determined using an amidolytic anti Xa assay. The control values for NHP and NMP were 0 % inhibition. Data represents a mean ± S.E. The experiments were performed five times on separate days.

IN VITRO ANTIPROTEASE ACTIVITY OF HEPARIN AND CY 216
AFTER PROTAMINE ADMINISTRATION



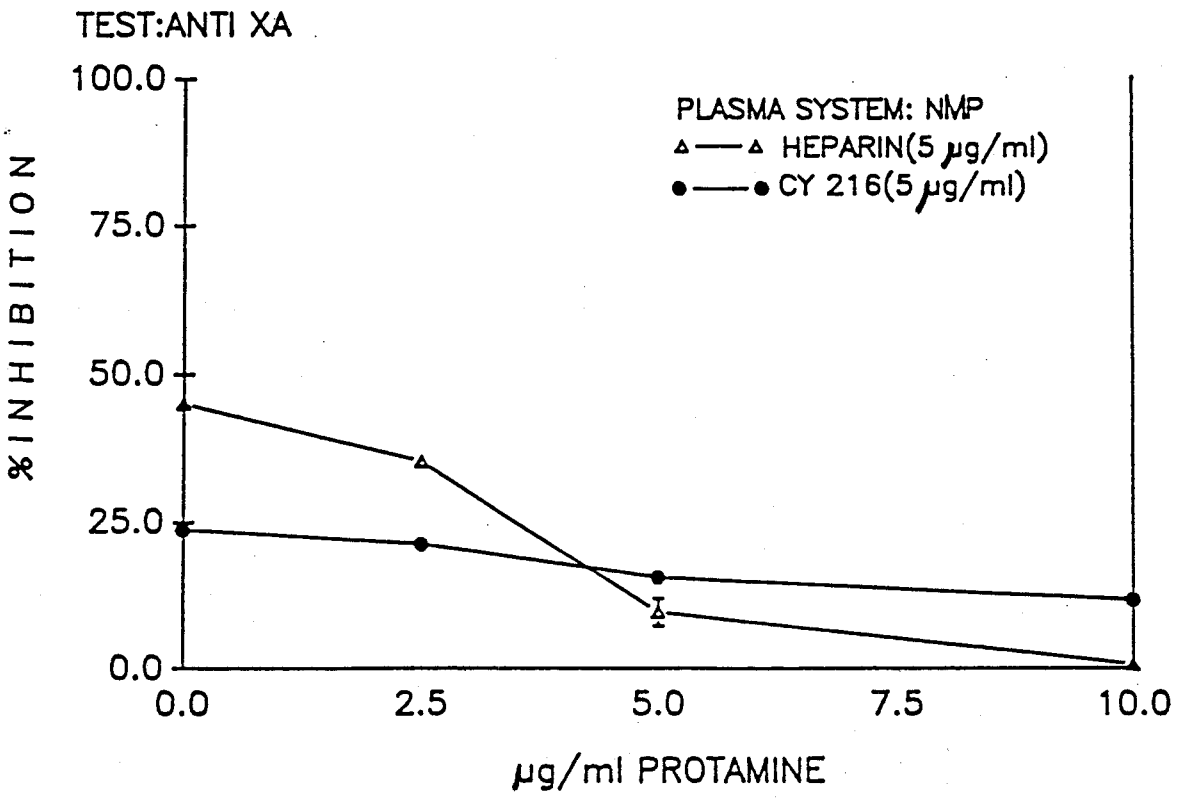
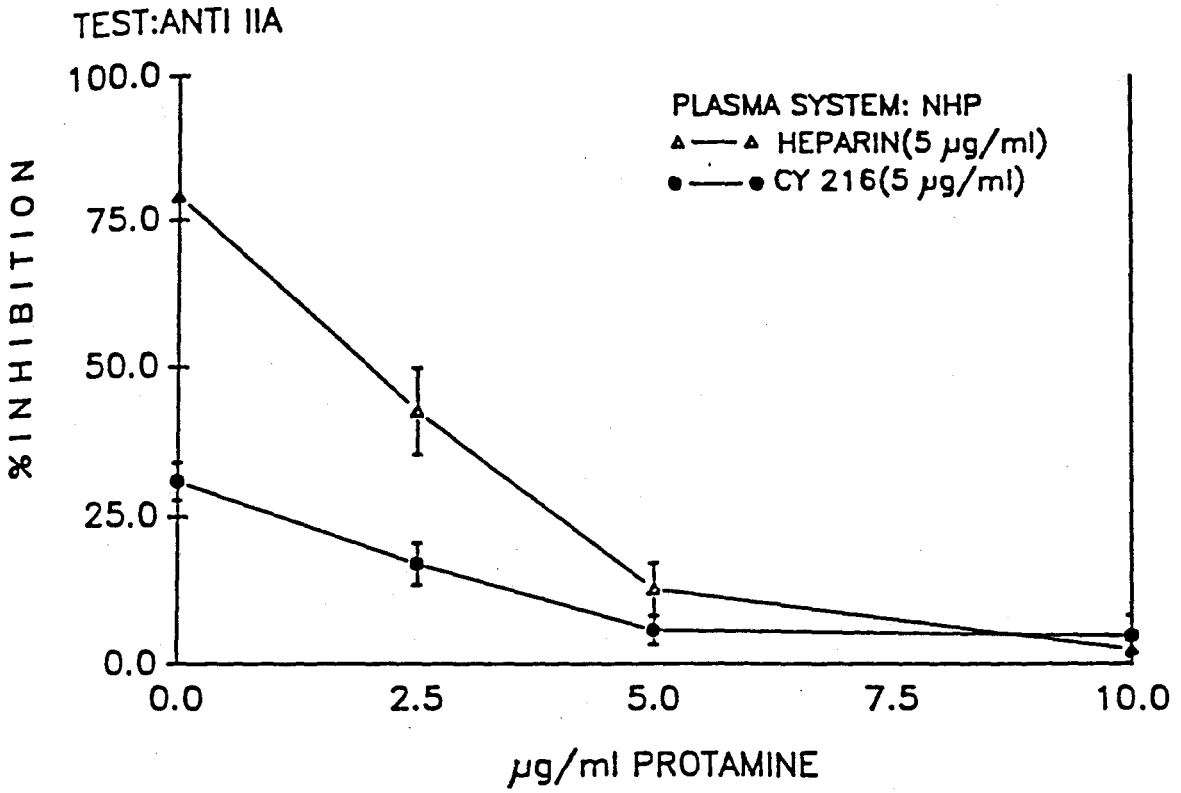
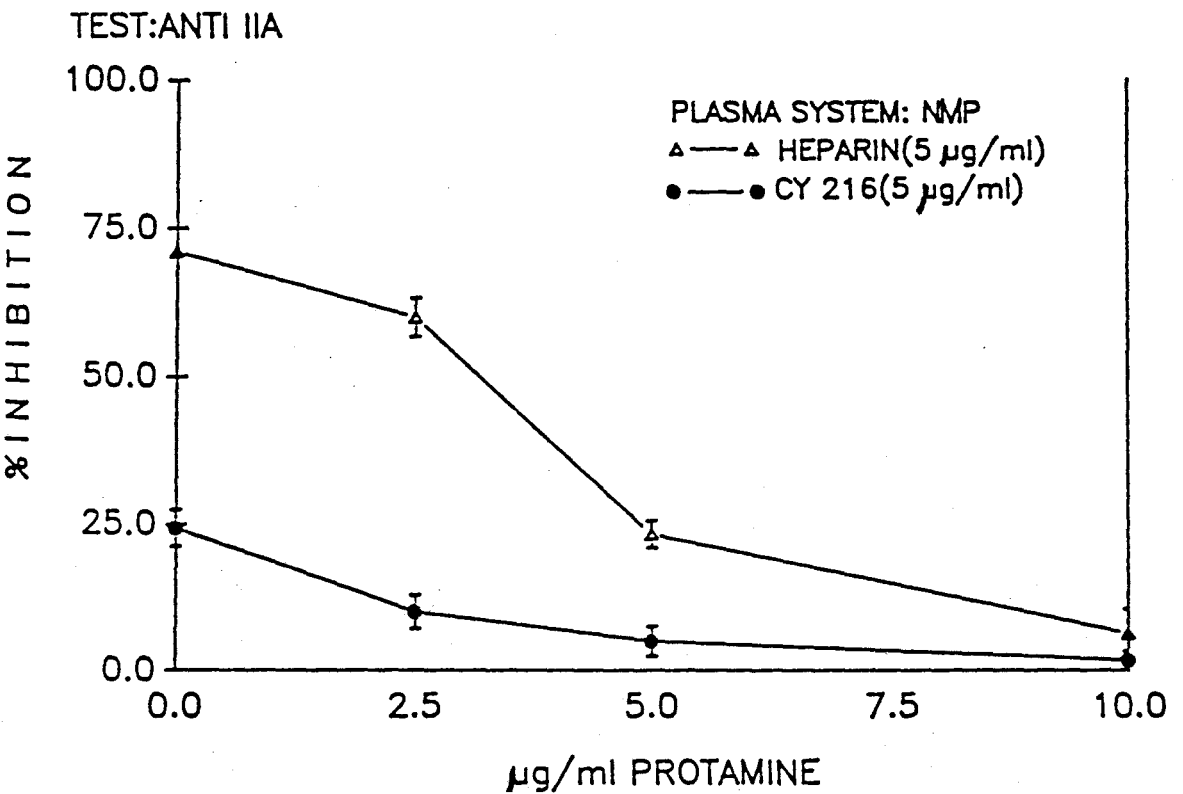


Figure 13. Heparin or CY 216 was supplemented to NHP (upper panel) or to NMP to obtain a final concentration of 5 μg/ml. Protamine sulfate was added to the heparin and CY 216 solutions to obtain final protamine concentrations of 2.5 μg/ml, 5.0 μg/ml and 10 μg/ml. Antiprotease activity was determined using an amidolytic anti IIa assay. The control values for NHP and NMP were 0 % inhibition. Data represents a mean ± S.E. The experiments were performed five times on separate days.

IN VITRO ANTIPROTEASE ACTIVITY OF HEPARIN AND CY 216
AFTER PROTAMINE ADMINISTRATION





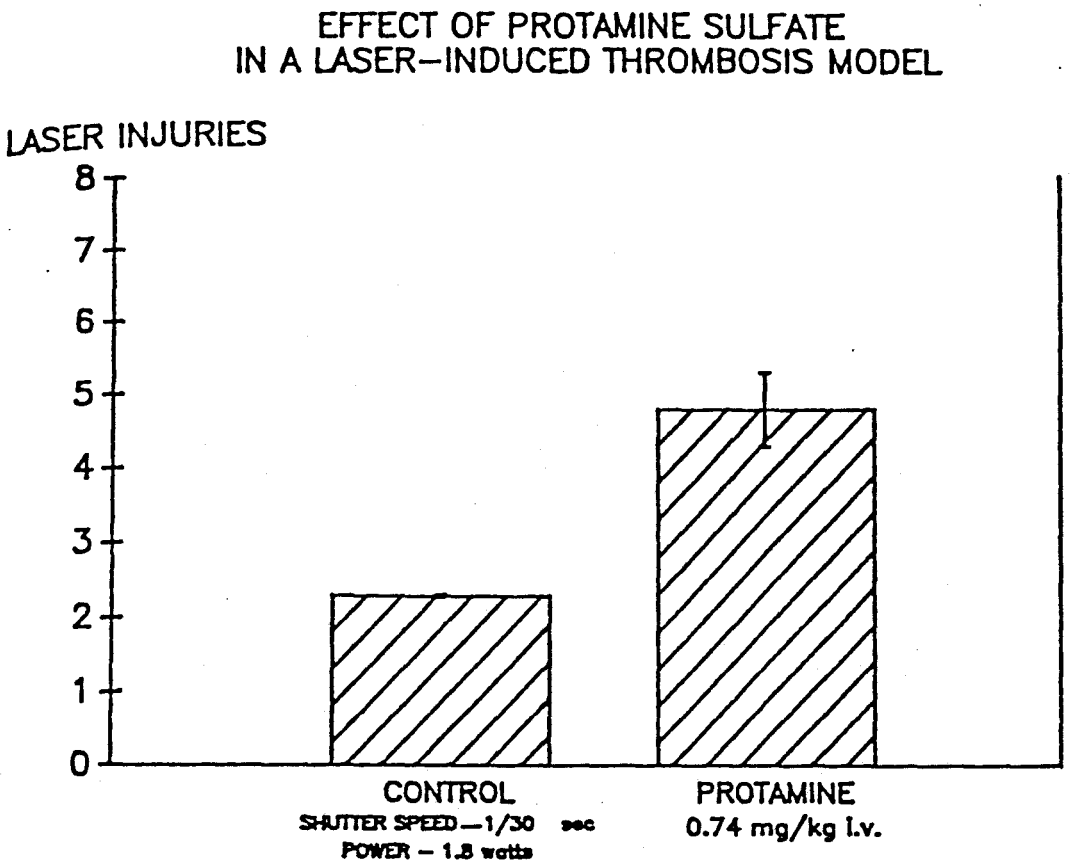


Figure 14. Two groups of rats were used in this study. Group I received a laser injury consisting of 0.18 Watts for 1/30 second. Protamine sulfate (0.74 mg/kg i.v.) was administered to the second group of rats via the tail vein, 15 minutes prior to laser injury. The number of laser injuries needed to produce a thrombus at least as long and as broad of the diameter of the vessel was recorded. Data represents a mean ± S.E. N = 11 for the control group. N = 3 for protamine (0.74 mg/kg i.v.) group. Statistical analysis was performed by an unpaired student's t test:
t= 1.110, df=13, p>.20, N.S.

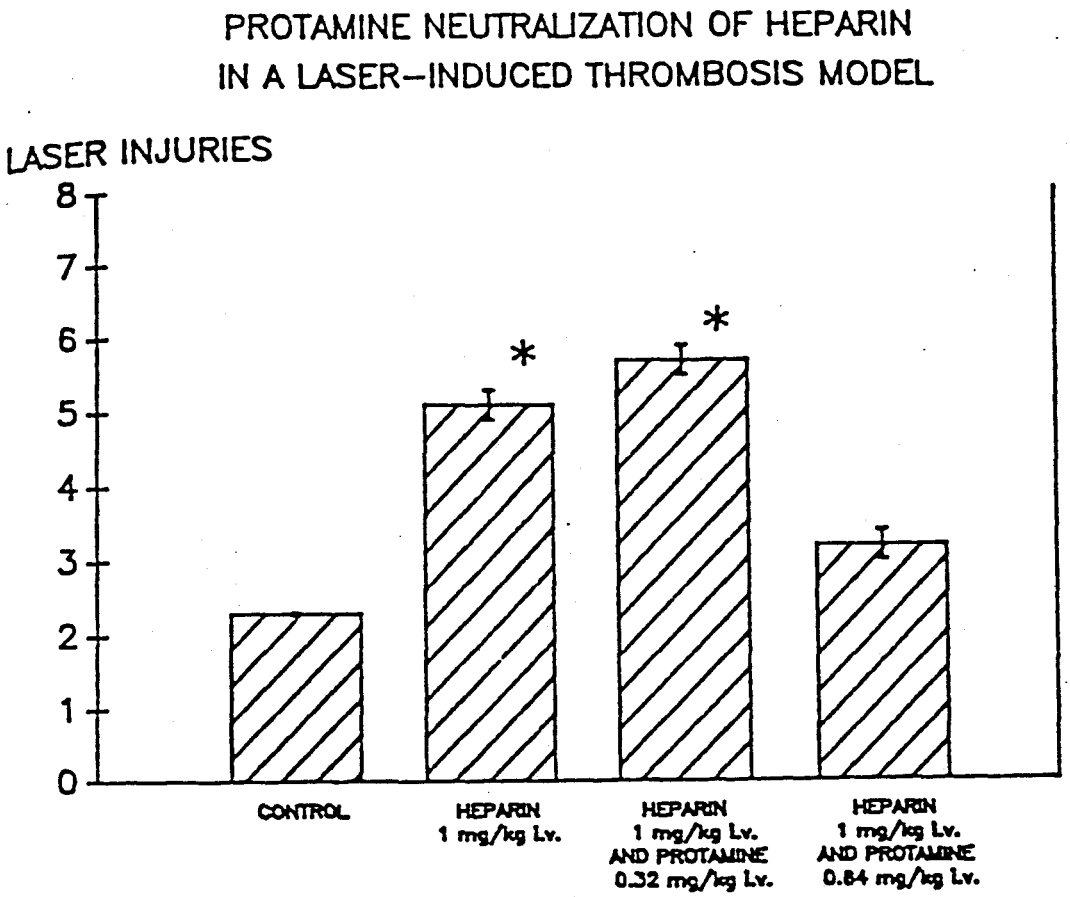


Figure 15. Heparin (1 mg/kg i.v.) was administered to 3 groups of rats via the tail vein 15 minutes prior to laser injury. Two groups of rats received either protamine (0.32 or 0.64 mg/kg i.v.), 5 minutes after heparin and was allowed to circulate for 10 minutes. Immediately after, a laser injury was inflicted at the mesenteric vessel wall. The number of laser injuries needed to produce the standard thrombus was recorded. Data represents a mean ± S.E. N = 11 for control group. N = 4 for heparin (1 mg/kg i.v.) group. N = 6 for heparin (1 mg/kg i.v.) and protamine (0.32 mg/kg i.v.) group. N = 5 for heparin (1 mg/kg i.v). and protamine (0.64 mg/kg i.v.) group. Statistical analysis was performed by a one way ANOVA: $F(3,22) = 26.7555$, $p < .05$ and followed by a Newman-Keuls multiple comparison test. * represents a significant difference from control the group.

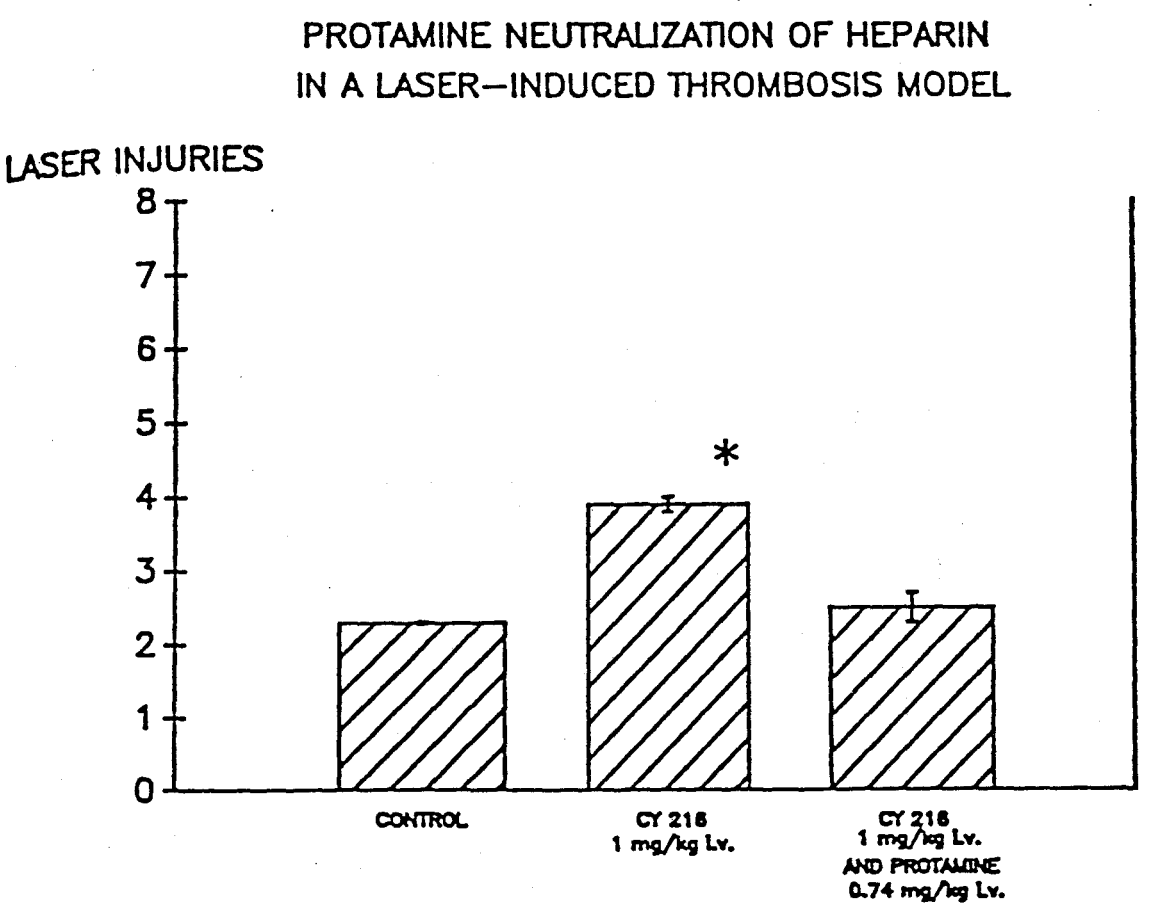


Figure 16. CY 216 (1 mg/kg i.v.) was administered to rats via the tail vein 15 minutes prior to laser injury. One group of rats received protamine (0.74 mg/kg i.v.) 5 minutes after CY 216 and was allowed to circulate for 10 minutes. Immediately after a laser injury was inflicted at the mesenteric vessel wall. The number of laser injuries needed to produce the standard thrombus was recorded. Data represents a mean ± S.E. N = 11 for control group. N = 5 for CY 216 (1 mg/kg i.v.) group. N = 7 for CY 216 (1 mg/kg i.v.) and protamine (0.74 mg/kg i.v.) group. Statistical analysis was performed by a one way ANOVA: $F(2,20) = 6.608$, $p < .05$ and followed by a Newman-Keuls multiple comparison test. * represents a significant difference from the control group.

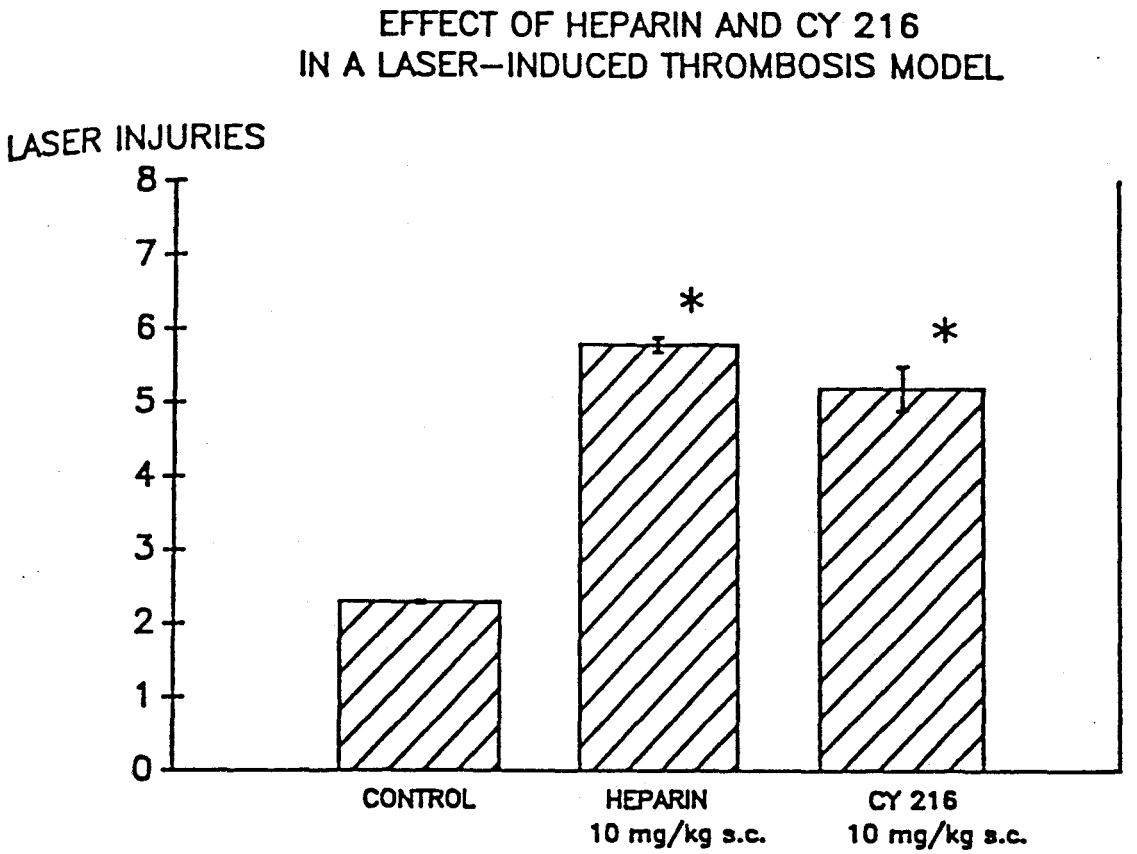


Figure 17. Heparin (10 mg/kg s.c.) or CY 216 (10 mg/kg s.c.) was administered to rats 2 hours prior to laser injury. The number of laser injuries needed to produce the standard thrombus was recorded. Data represents a mean ± S.E. N = 11 for control group. N = 5 for heparin (10 mg/kg s.c.) group and N = 5 for CY 216 (10 mg/kg s.c.) group. Statistical analysis was performed by a one way ANOVA: $F(2,18) = 47.681$, $p < .05$ and followed by a Newman-Keuls multiple comparison test.
* represents significant difference from control group.

CLOT SCORES OBTAINED IN A RABBIT STASIS THROMBOSIS MODEL AFTER PROTAMINE ADMINISTRATION

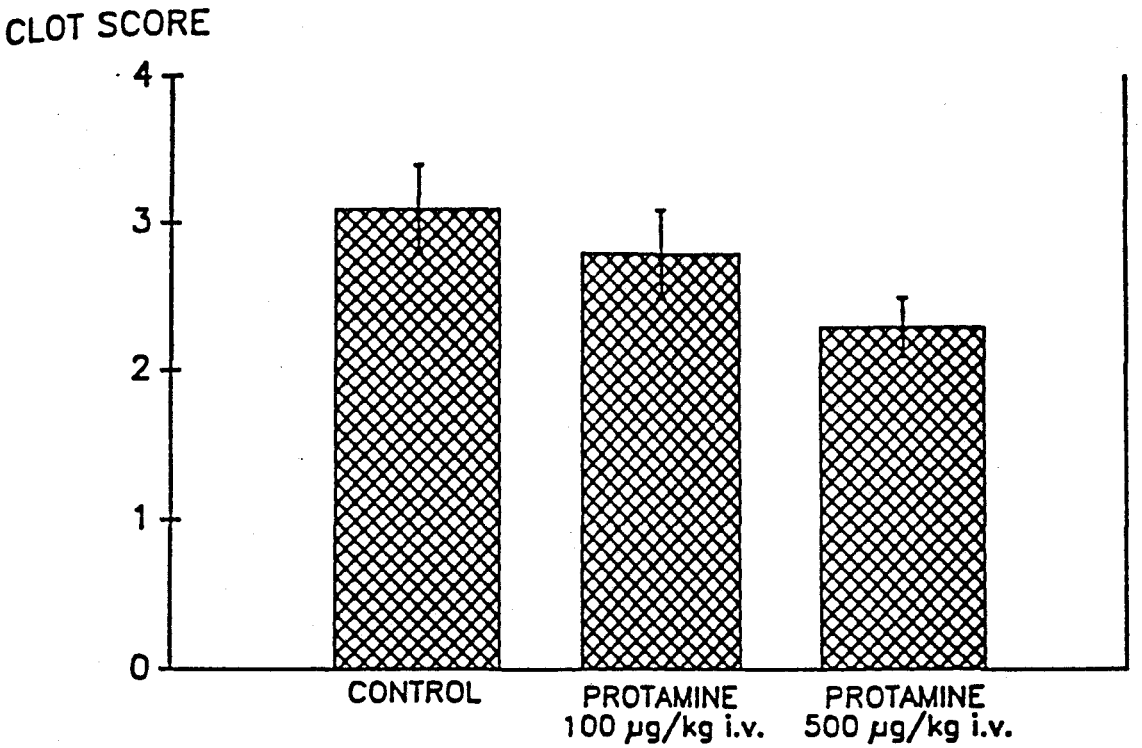


Figure 18. Rabbits were anaesthetized and segments of the rabbit jugular veins were surgically exposed. Protamine (100 or 500 µg/kg i.v.) or an equivalent volume of saline was administered via the rabbit ear vein and allowed to circulate for 10 minutes at which time, blood was drawn by cardiac puncture and a thrombogenic challenge comprised of PCC/RVV was given. After exactly 20 seconds, both jugular veins were ligated for 10 minutes, excised and evaluated for degree of clot formation as described in the methods section. Data represents a mean ± S.E. N = 5 for control group. N = 3 for protamine (100 µg/kg i.v.) group. N = 3 for protamine (500 µg/kg i.v.) group. Statistical analysis was performed by a Kruskal-Wallis test: H = 2.610, $p > .10$, N.S.

CLOT SCORES OBTAINED IN A RABBIT STASIS THROMBOSIS MODEL AFTER HEPARIN ADMINISTRATION

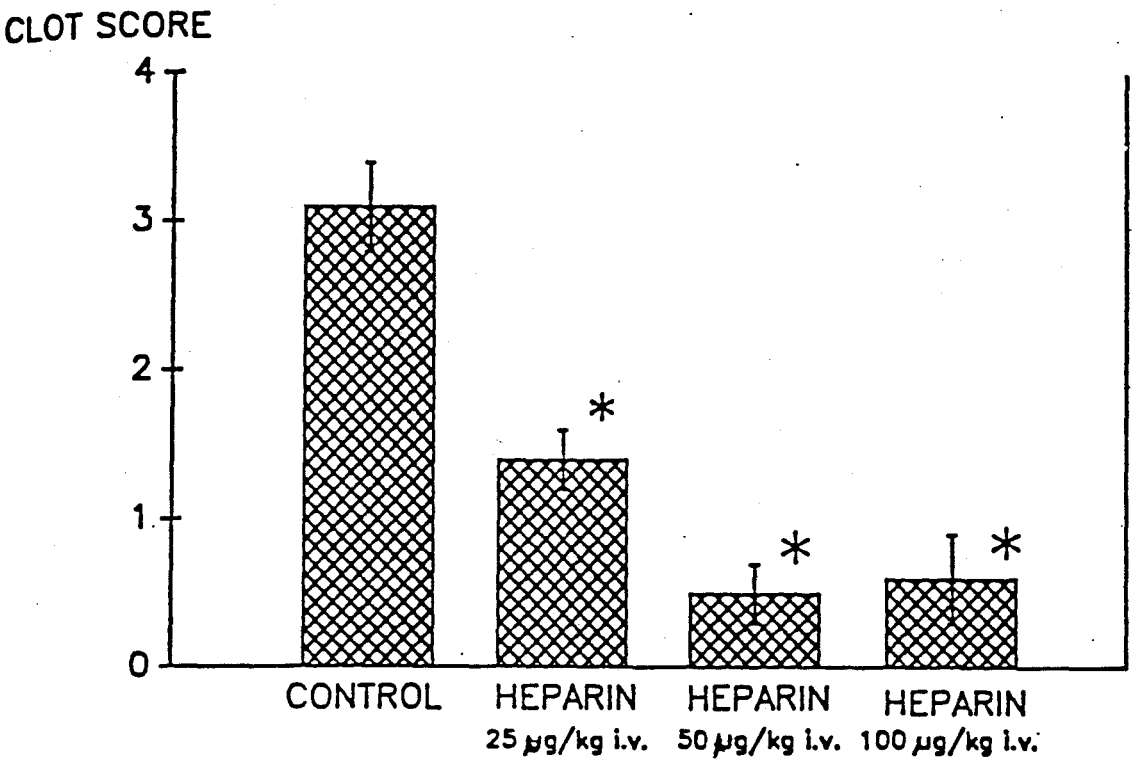


Figure 19. Four groups consisting of five rabbits each were anaesthetized and segments of the jugular veins were surgically exposed. Heparin (25 µg/kg i.v.), heparin (50 µg/kg i.v.) or heparin (100 µg/kg i.v.) or an equivalent volume of saline was administered via the ear vein and allowed to circulate for 10 minutes at which time a thrombogenic challenge comprised of PCC/RVV was given. After exactly 20 seconds both jugular veins were ligated for 10 minutes, excised and evaluated for degree of clot formation. Data represents a mean ± S.E. Statistical analysis was performed by a Kruskal-Wallis test: $H = 14.308$, $p < .05$ and followed by a nonparametric Tukey multiple comparison test. * represents a significant difference from control group.

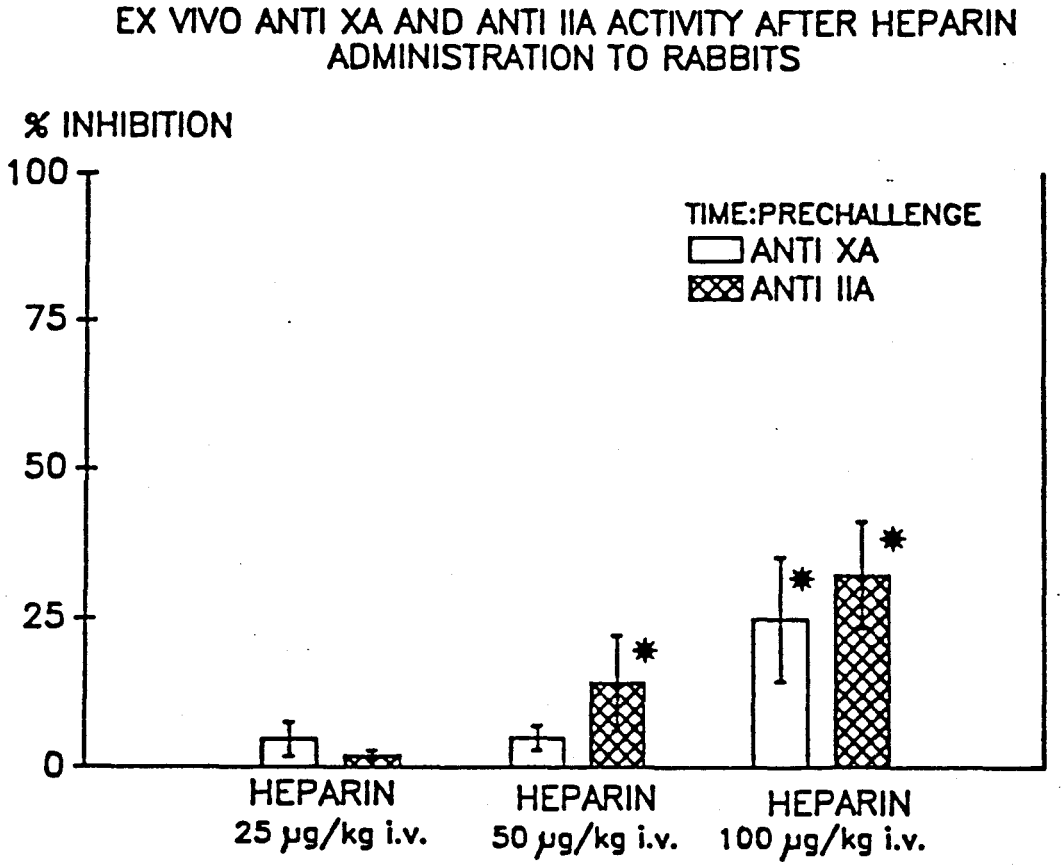


Figure 20. Three groups consisting of five rabbits each were anaesthetized and segments of the jugular veins were surgically exposed. Heparin (25 µg/kg i.v.), heparin (50 µg/kg i.v.) or heparin (100 µg/kg i.v.) or an equivalent volume of saline was administered via the ear vein and allowed to circulate for 10 minutes at which time blood was drawn by cardiac puncture. Antiprotease activities were determined using amidolytic anti Xa and amidolytic anti IIa assays. Control values for normal rabbit plasma were 0 % inhibition. Data represents a mean ± S.E. Statistical analysis was performed by a two way ANOVA:

Factor A (heparin dose): $F(2,24) = 10.059$ $p < .05$

Factor B (test): $F(1,24) = 0.672$ $p > .50$ N.S.

Factor A x B: $F(2,24) = 0.444$ $p > .50$ N.S.

followed by Newman-Keuls multiple comparison tests. * represents a significant difference from the control group.

CLOT SCORES OBTAINED IN A RABBIT STASIS THROMBOSIS MODEL AFTER CY 216 ADMINISTRATION

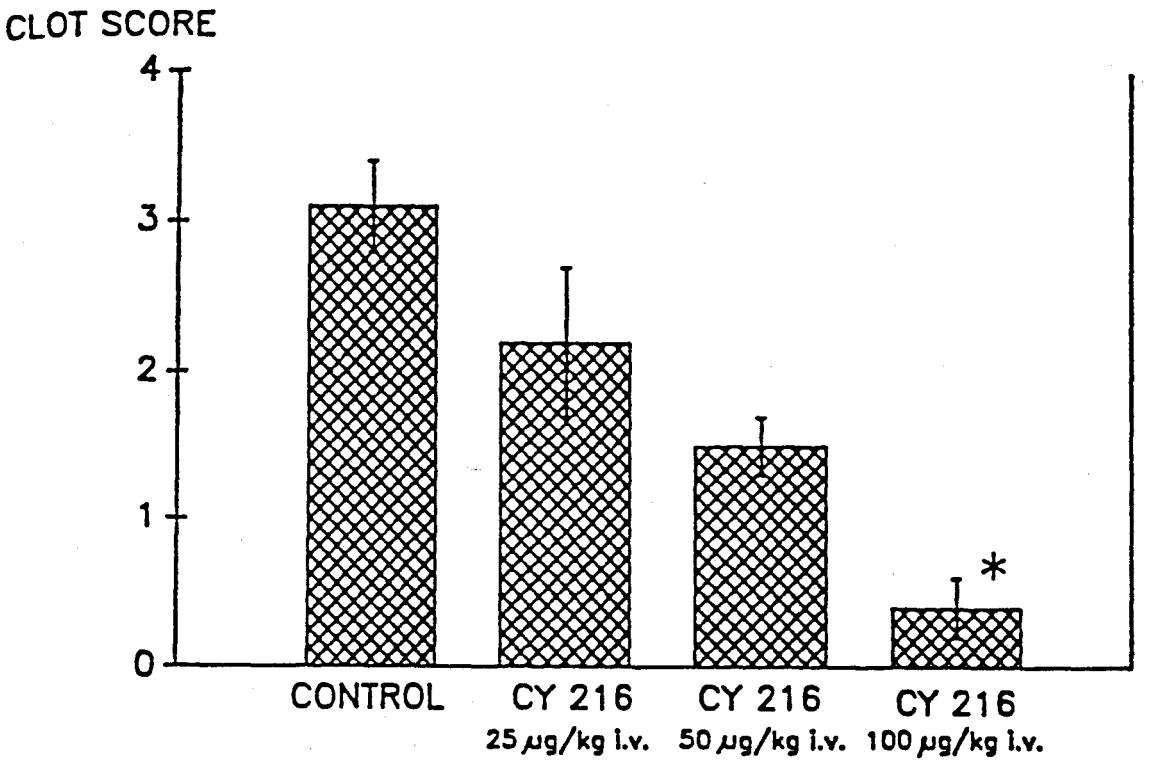


Figure 21. Four groups consisting of five rabbits each were anaesthetized and segments of the jugular veins were surgically exposed. CY 216 (25 µg/kg i.v.), CY 216 (50 µg/kg i.v.) or CY 216 (100 µg/kg i.v.) or an equivalent volume of saline was administered via the ear vein and allowed to circulate for 10 minutes at which time a thrombogenic challenge comprised of PCC/RVV was given. After exactly 20 seconds both jugular veins were ligated for 10 minutes, excised and evaluated for degree of clot formation. Data represents a mean ± S.E. Statistical analysis was performed by a Kruskal-Wallis test: $H = 26.988$, $p < .05$ and followed by a nonparametric Tukey multiple comparison test. * represents a significant difference from control group.

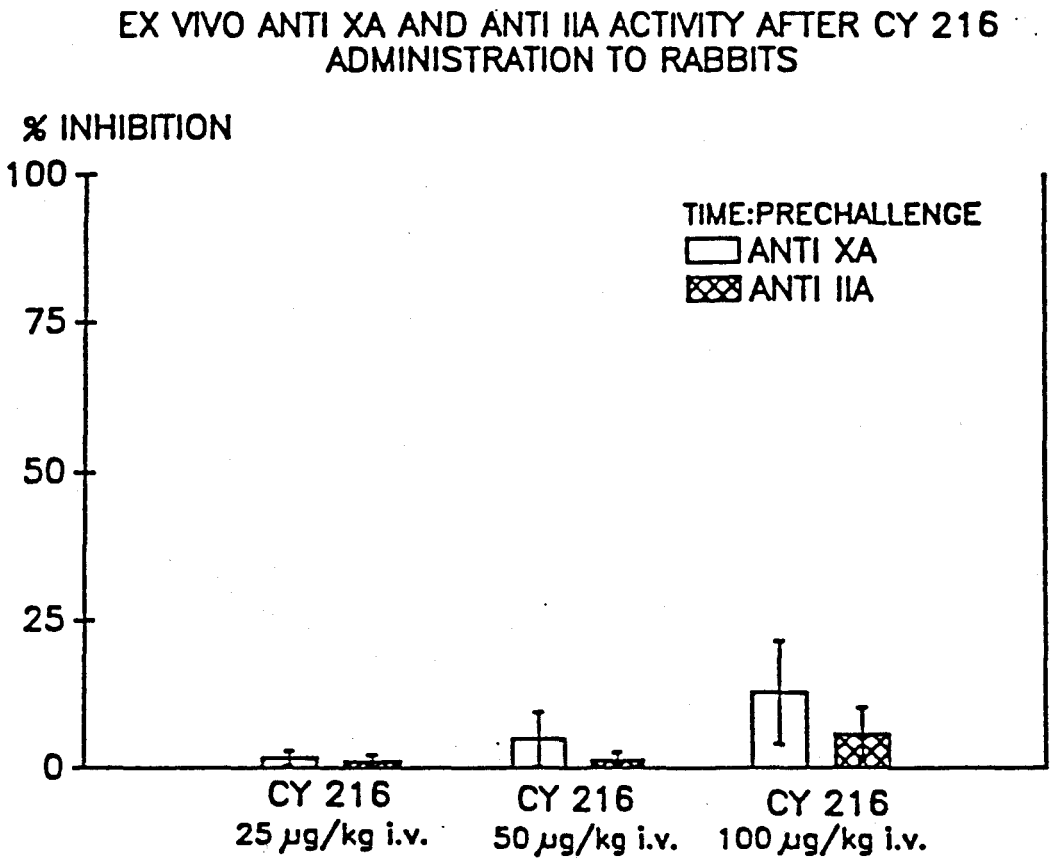


Figure 22. Three groups consisting of five rabbits each were anaesthetized and segments of the jugular veins were surgically exposed. CY 216 (25 µg/kg i.v.), CY 216 (50 µg/kg i.v.) or CY 216 (100 µg/kg i.v.) or an equivalent volume of saline was administered via the ear vein and allowed to circulate for 10 minutes at which time blood was drawn by cardiac puncture. Antiprotease activities were determined using the amidolytic anti Xa and amidolytic anti IIa assays. Control values for normal rabbit plasma was 0% inhibition. Data represents a mean ± S.E. Statistical analysis were performed by a two way ANOVA:

Factor A (CY 216 dose): $F(2,24) = 1.070$ $p > .50$ N.S.
Factor B (test): $F(1,24) = 0.843$ $p > .50$ N.S.
Factor A x B: $F(2,24) = 1.408$ $p > .50$ N.S.

CLOT SCORES OBTAINED IN A RABBIT STASIS THROMBOSIS MODEL

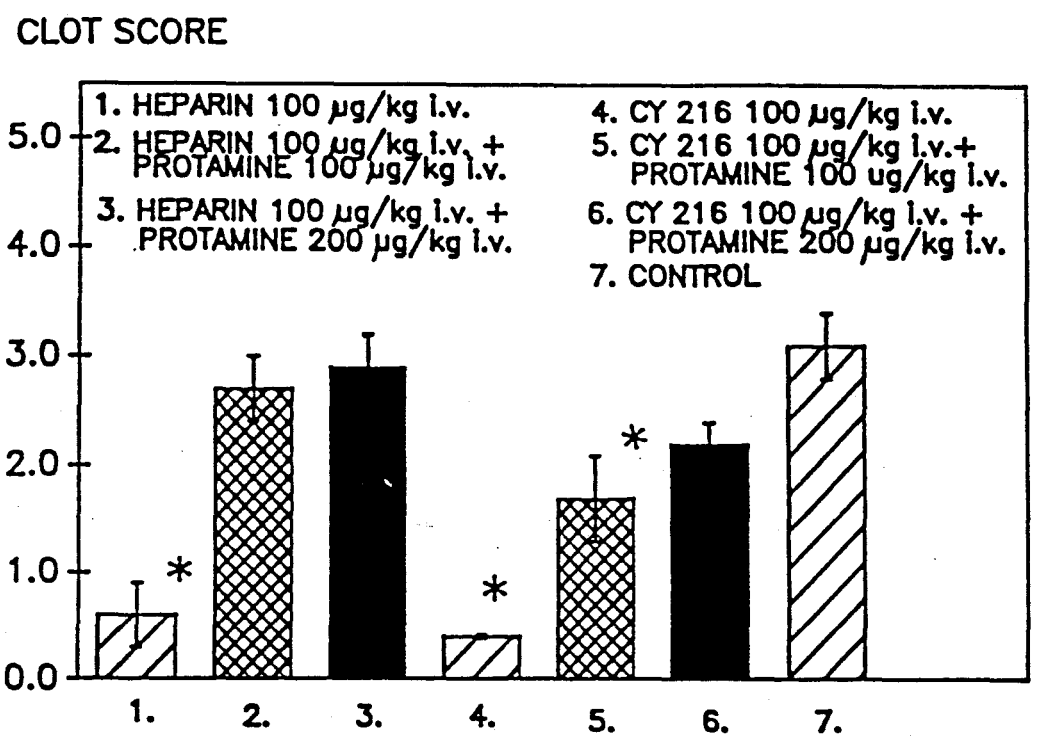


Figure 23. Seven groups consisting of five rabbits each were anaesthetized and segments of the jugular veins were surgically exposed. Heparin (100 µg/kg i.v.) or CY 216 (100 µg/kg i.v.) was administered via the ear vein and allowed to circulate for 10 minutes and blood was drawn by cardiac puncture. To groups of rabbits which received protamine (100 µg/kg i.v. or 200 µg/kg i.v.), this drug was administered 5 minutes after heparin (100 µg/kg i.v.) or CY 216 (100 µg/kg i.v.) and allowed to circulate for 5 minutes at which time blood was drawn. Immediately after blood draw, a thrombogenic challenge comprised of PCC/RVV was given. After exactly 20 seconds both jugular veins were ligated for 10 minutes, excised and evaluated for degree of clot formation. Data represents a mean ± S.E. Statistical analysis was performed by a two way ANOVA:

Factor A (drug treatment): $F(2,36) = 18.049$ $p < .05$
Factor B (protamine dose): $F(2,36) = 16.810$ $p > .20$ N.S.
Factor A x B: $F(4,36) = 5.453$ $p < .05$

followed by a Newman-Keuls multiple comparison test. * represents a significiacant difference from the control group.

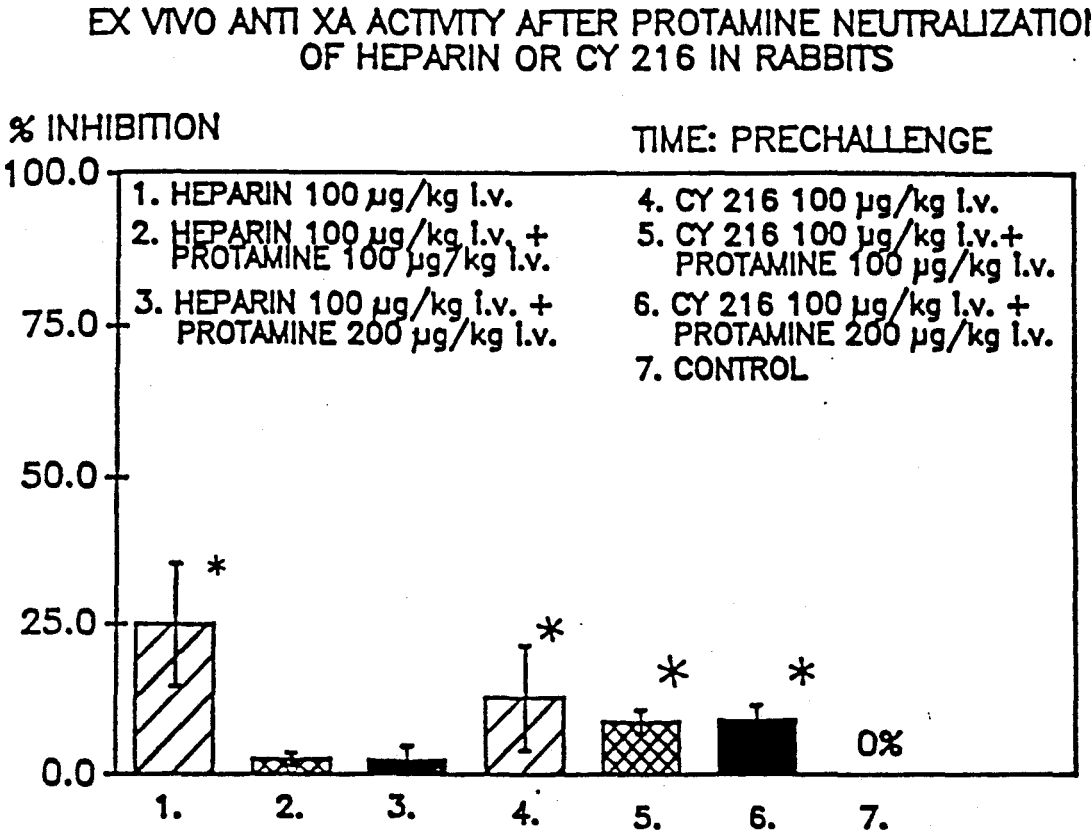


Figure 24. Seven groups consisting of five rabbits each were anaesthetized and segments of the jugular veins were surgically exposed. Heparin (100 µg/kg i.v.) or CY 216 (100 µg/kg i.v.) was administered via the ear vein and allowed to circulate for 10 minutes and blood was drawn by cardiac puncture. To groups of rabbits which received protamine (100 µg/kg i.v. or 200 µg/kg i.v.), this drug was administered 5 minutes after heparin (100 µg/kg i.v.) or CY 216 (100 µg/kg i.v.) and allowed to circulate for 5 minutes at which time blood was drawn. Antiprotease activity was determined using an amidolytic anti Xa assay. Data represents a mean ± S.E. Statistical analysis was performed by a two way ANOVA:

Factor A (drug treatment): $F(2,36) = 5.167$ $p < .05$
Factor B (protamine dose): $F(2,36) = 1.635$ $p > .20$ N.S.
Factor A x B: $F(4,36) = 5.104$ $p < .05$

followed by a Newman-Keuls multiple comparison test. * represents a significacant difference from the control group.

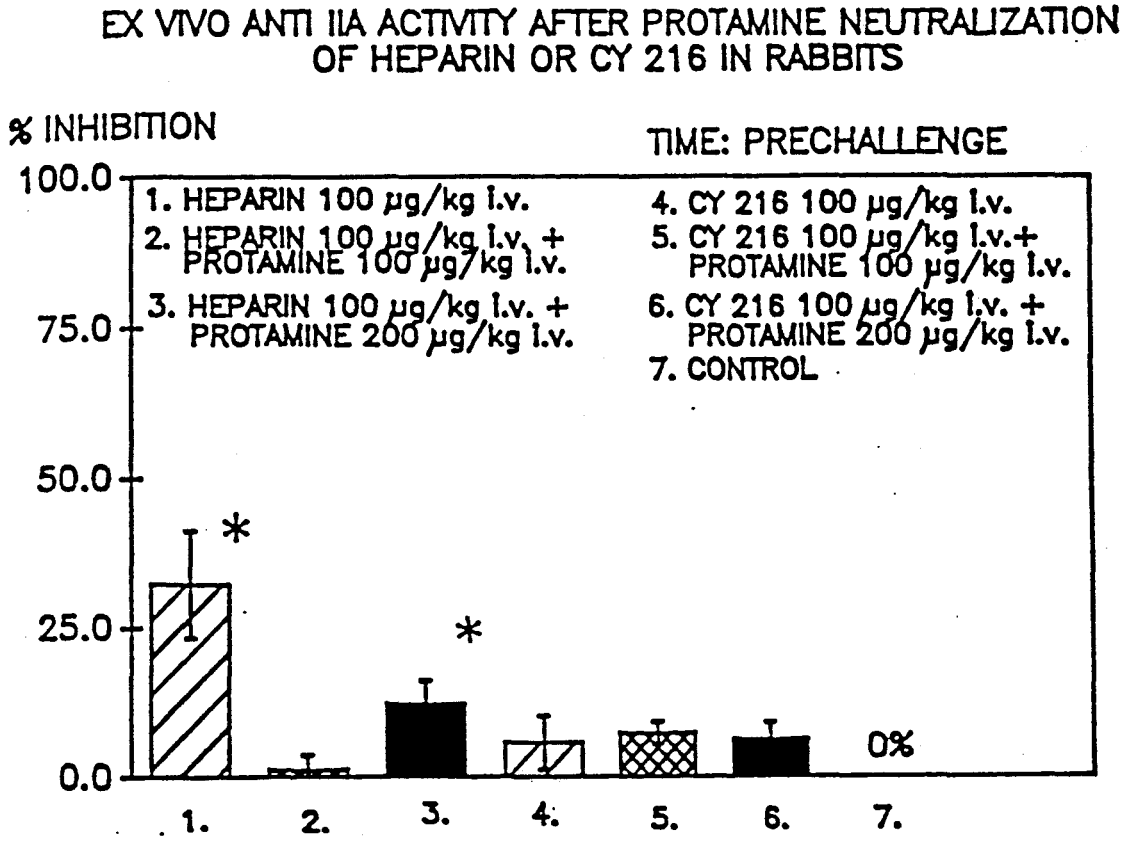


Figure 25. Seven groups consisting of five rabbits each were anaesthetized and segments of the jugular veins were surgically exposed. Heparin (100 µg/kg i.v.) or CY 216 (100 µg/kg i.v.) was administered via the ear vein and allowed to circulate for 10 minutes and blood was drawn by cardiac puncture. To groups of rabbits which received protamine (100 µg/kg i.v. or 200 µg/kg i.v.), this drug was administered 5 minutes after heparin (100 µg/kg i.v.) or CY 216 (100 µg/kg i.v.) and allowed to circulate for 5 minutes at which time blood was drawn. Antiprotease activity was determined using an amidolytic anti IIa assay. Data represents a mean ± S.E. Statistical analysis was performed by a two way ANOVA:

Factor A (drug treatment): $F(2,36) = 25.553$ $p < .05$
Factor B (protamine dose): $F(2,36) = 16.684$ $p < .05$
Factor A x B: $F(4,36) = 4.850$ $p < .05$

followed by a Newman-Keuls multiple comparison test. * represents a significant difference from control group.

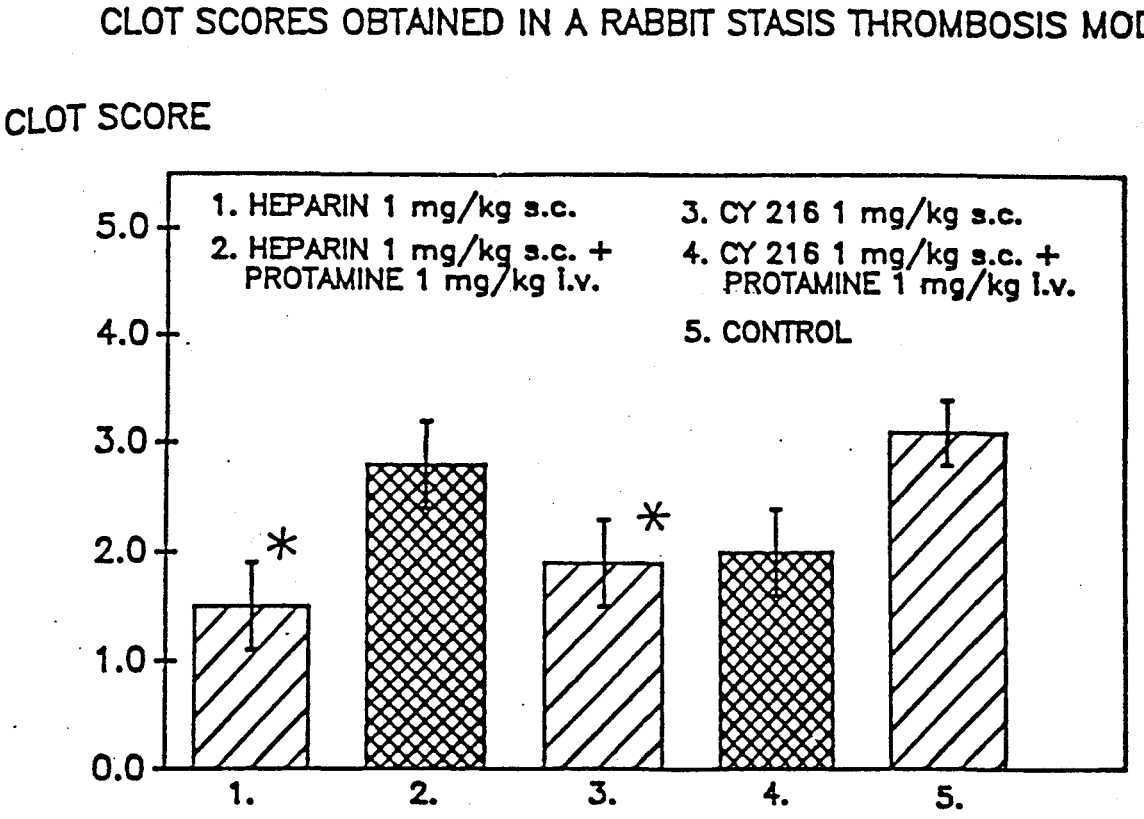


Figure 26. Five groups consisting of five rabbits each were anaesthetized and segments of the jugular veins were surgically exposed. Heparin (1 mg/kg s.c.) or CY 216 (1 mg/kg s.c.) was administered and allowed to circulate for 2 hours. Blood was drawn by cardiac puncture. To groups of rabbits which received protamine (1 mg/kg i.v.), this drug was administered 2 hours after heparin (1 mg/kg s.c.) or CY 216 (1 mg/kg s.c.) and allowed to circulate for 5 minutes at which time blood was drawn. Immediately after blood draw, a thrombogenic challenge comprised of PCC/RVV was given. After exactly 20 seconds both jugular veins were ligated for 10 minutes, excised and the jugular vein segments were evaluated for degree of clot formation. Data represents a mean ± S.E. Statistical analysis was performed by a two way ANOVA:

Factor A (drug treatment): $F(2,24) = 5.298$ $p < .05$
Factor B (protamine dose): $F(2,24) = 1.739$ $p > .20$ N.S.
Factor A x B: $F(4,24) = 5.428$ $p < .05$

followed by a Newman Keuls multiple comparison test. * represents a significant difference from the control group.

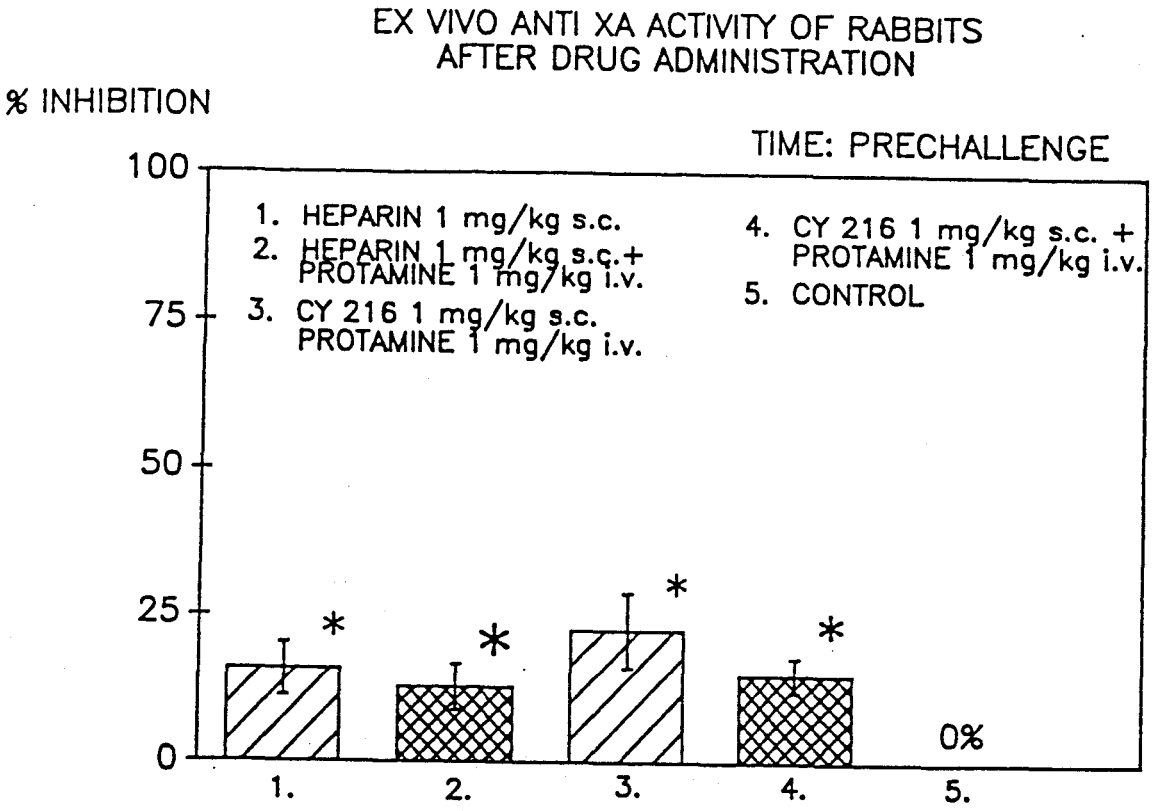


Figure 27. Five groups consisting of five rabbits each were anaesthetized and segments of the jugular veins were surgically exposed. Heparin (1 mg/kg s.c.) or CY 216 (1 mg/kg s.c.) was administered and allowed to circulate for 2 hours at which time blood was drawn by cardiac puncture. To groups of rabbits which received protamine (1 mg/kg i.v.), this drug was administered 2 hours after heparin (1 mg/kg s.c.) or CY 216 (1 mg/kg s.c.) and allowed to circulate for 5 minutes at which time blood was drawn. Antiprotease activity was determined using an amidolytic anti Xa assay. Statistical analysis was performed by a two way ANOVA:

Factor A (drug treatment): $F(2,24) = 13.401$ $p < .05$
Factor B (protamine dose): $F(1,24) = 6.079$ $p < .05$
Factor A x B: $F(2,24) = 6.557$ $p < .05$

followed by a Newman-Keuls multiple comparison test. * represents a significant difference from the control group.

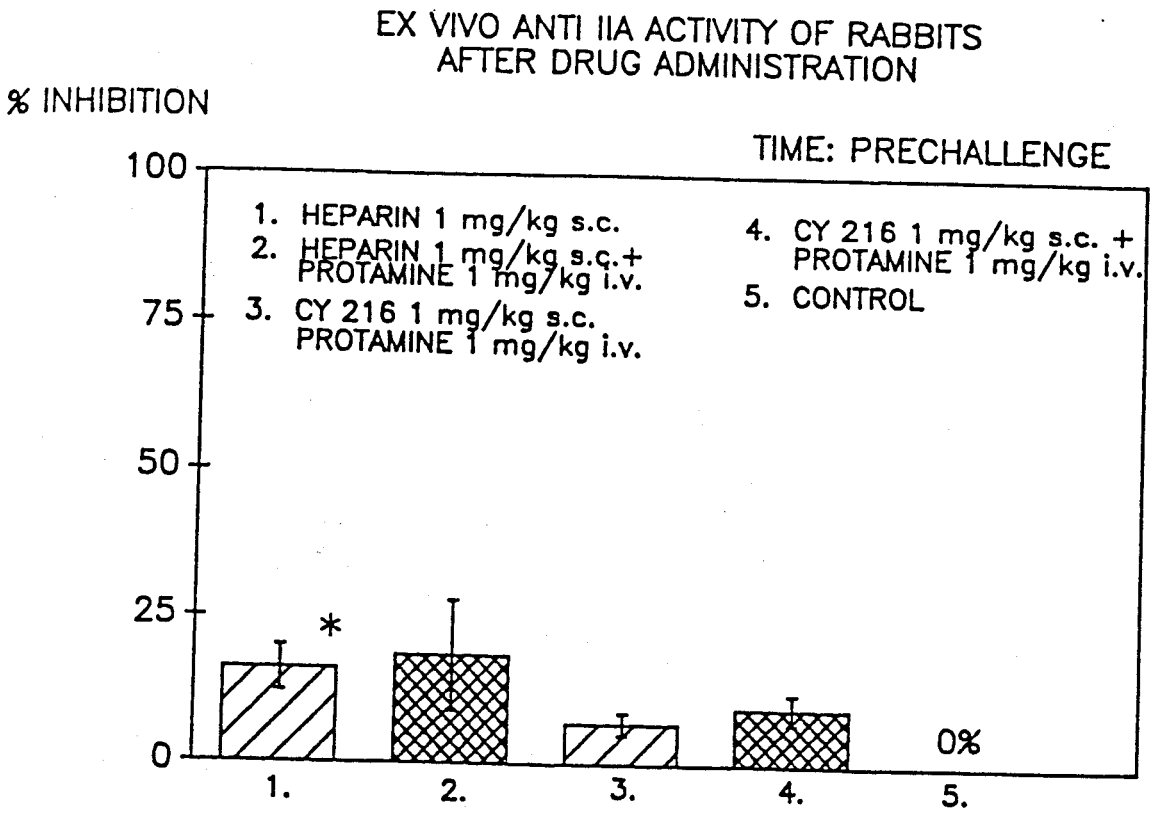


Figure 28. Five groups consisting of five rabbits each were anaesthetized and segments of the jugular veins were surgically exposed. Heparin (1 mg/kg s.c.) or CY 216 (1 mg/kg s.c.) was administered and allowed to circulate for 2 hours at which time blood was drawn by cardiac puncture. To groups of rabbits which received protamine (1 mg/kg i.v.), this drug was administered 2 hours after heparin (1 mg/kg s.c.) or CY 216 (1 mg/kg s.c.) and allowed to circulate for 5 minutes at which time blood was drawn. Antiprotease activity was determined using an amidolytic anti IIa assay. Statistical analysis was performed by a two way ANOVA:

Factor A (drug treatment): $F(2,24) = 8.446$ $p < .05$
Factor B (protamine dose): $F(1,24) = .620$ $p > .50$ N.S.
Factor A x B: $F(2,24) = .246$ $p > .50$ N.S.

followed by a Newman-Keuls multiple comparison test. * represents a significant difference from the control group.

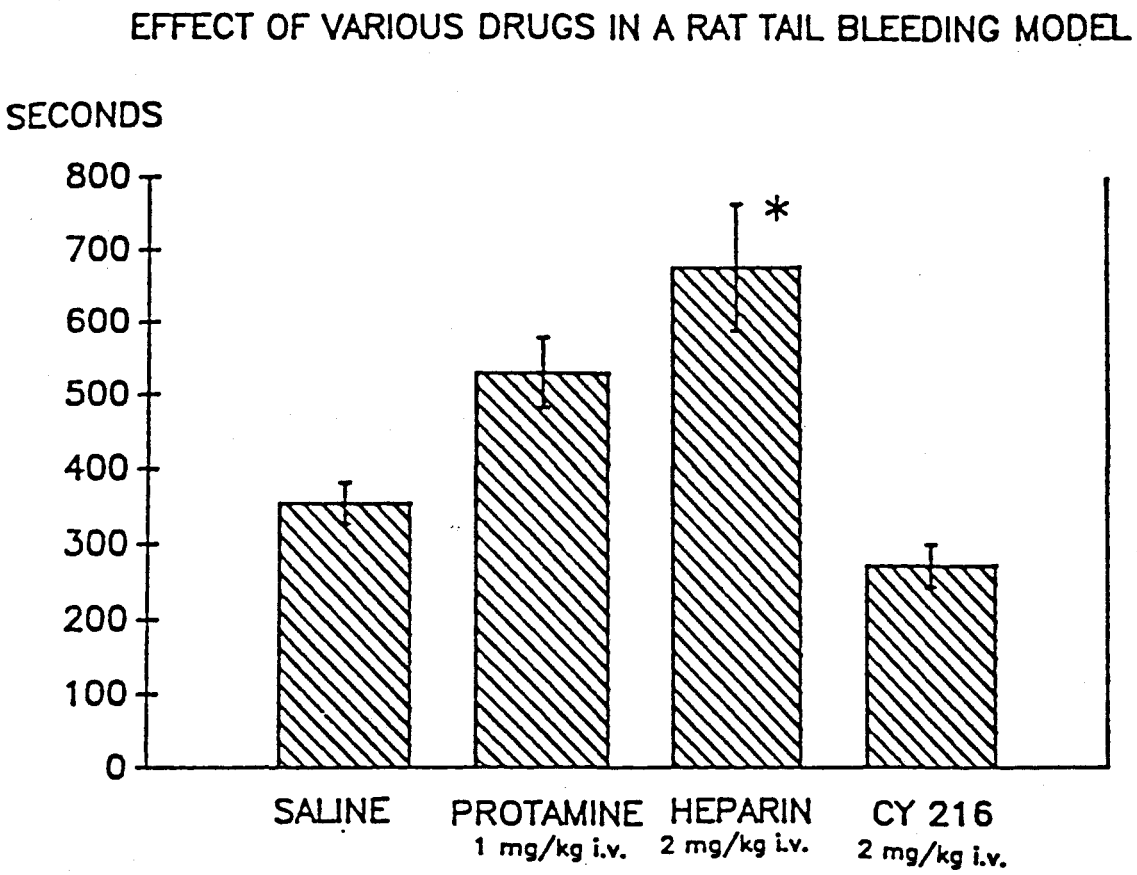


Figure 29. Groups of rats received either protamine (1 mg/kg i.v.), heparin (2 mg/kg i.v.), CY 216 (2 mg/kg i.v.) or an equivalent volume of saline via the tail vein. After 10 minutes bleeding time determinations were performed as described in the methods section. Data represents a mean ± S.E.M. N = 9 for saline group. N = 7 for protamine (1 mg/kg i.v.) group. N = 7 for heparin (2 mg/kg i.v.) group. N = 7 for CY 216 (2 mg/kg i.v.) group. Statistical analysis was performed by a one way ANOVA: $F(3,26) = 12.482$ $p < .05$ followed by a Newman-Keuls multiple comparison test: * represents a significant difference from CY 216 (2 mg/kg i.v.) group.

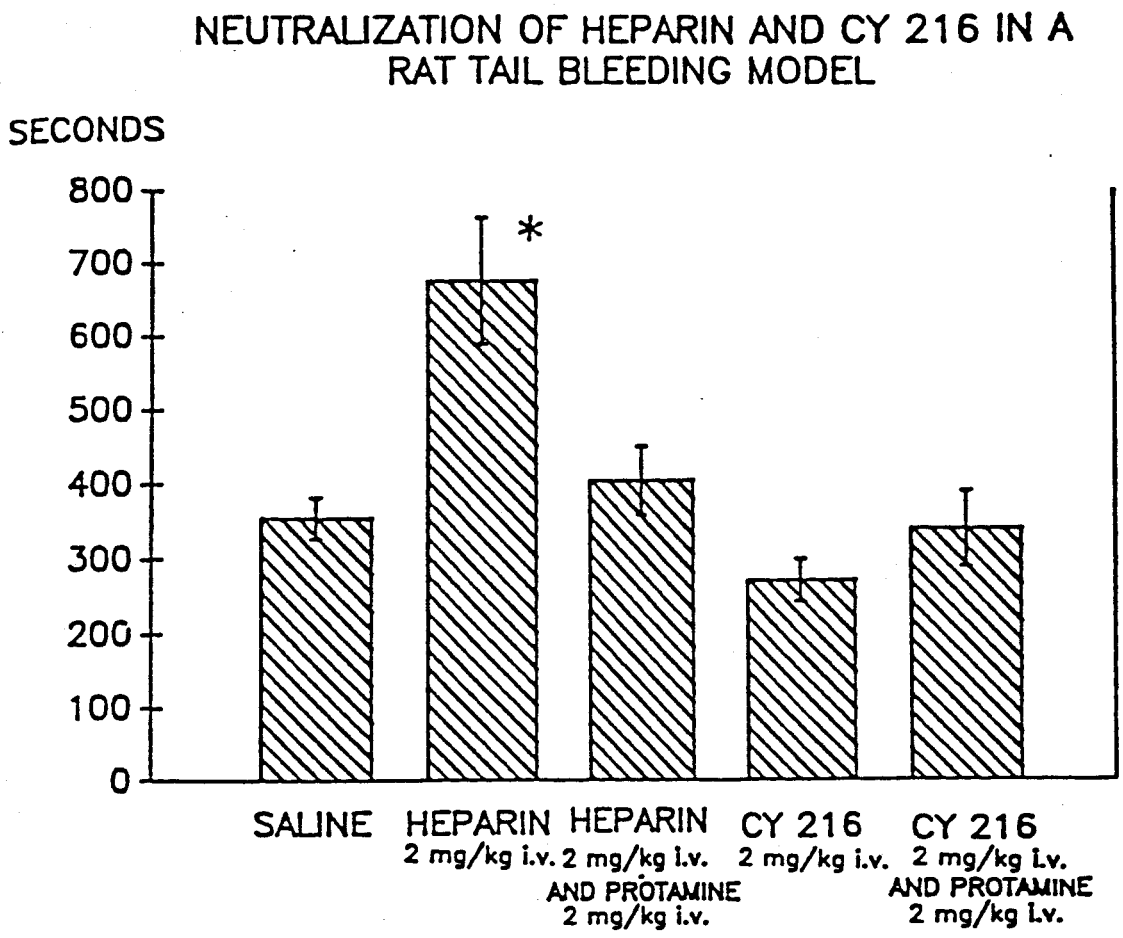


Figure 30. Groups of rats received either heparin (2 mg/kg i.v.), CY 216 (2 mg/kg i.v.) or an equivalent volume of saline via the tail vein. To rats which recieved protamine, this drug was administered 5 minutes after heparin or CY 216. After 10 minutes post heparin (or CY 216), bleeding time determinations were performed as described in the methods section. Data represents a mean ± S.E.M. N = 9 for saline group. N = 7 for heparin (2 mg/kg i.v.) group. N = 7 for CY 216 (2 mg/kg i.v.) group. N = 7 for heparin (2 mg/kg i.v.) and protamine (2 mg/kg i.v.) group. N = 7 for CY 216 (2 mg/kg i.v.) and protamine (2 mg/kg i.v.) group. Statistical analysis was performed by a one way ANOVA: $F(4,32) = 9.458$ $p < .05$ followed by a Newman-Keuls multiple comparison test: * represents a significant difference from CY 216 (2 mg/kg i.v.) group.

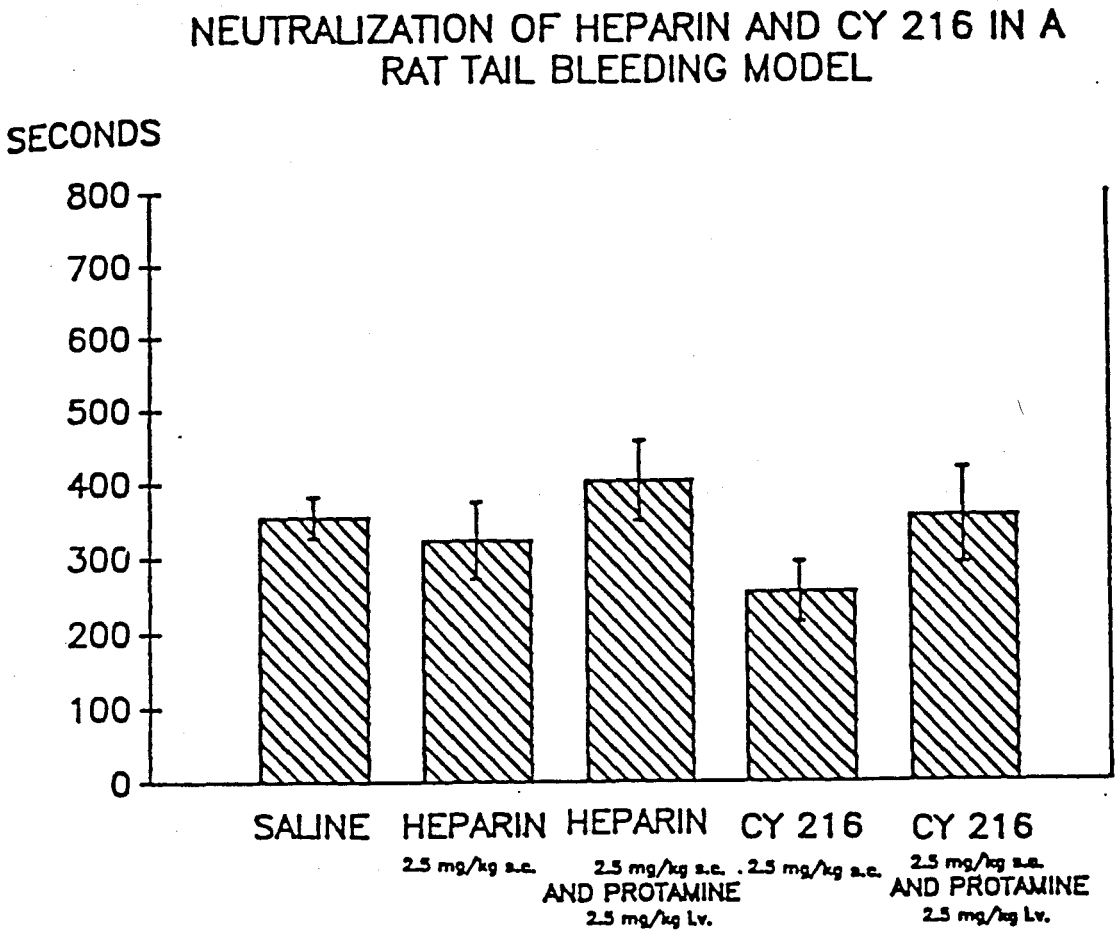


Figure 31. Groups of rats received heparin (2.5 mg/kg s.c.), CY 216 (2.5 mg/kg s.c.) or an equivalent volume of saline. Two groups of rats received protamine (2.5 mg/kg i.v.) in addition to heparin (2.5 mg/kg s.c.) or CY (2.5 mg/kg s.c.) After 120 minutes, bleeding time determinations were performed as described in the methods section. Data represents a mean ± S.E. N = 9 for saline group. N = 7 for all other groups. Statistical analysis was performed by a one way ANOVA: $F(4,32) = 1.294$ $p > .50$ N.S.

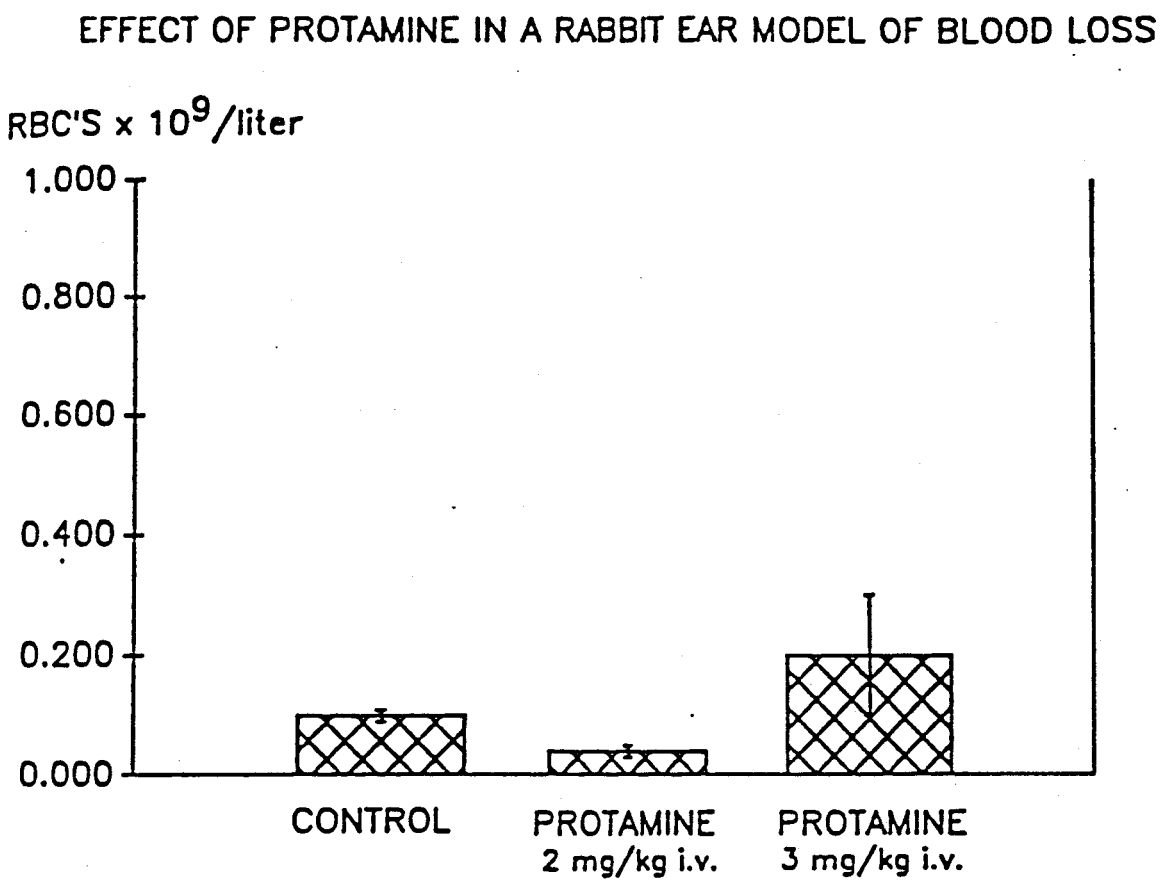


Figure 32. Individual groups of rabbits were administered either protamine (2 or 3 mg/kg i.v.) or an equivalent volume of saline which was allowed to circulate for 10 minutes. At this time, blood was drawn by cardiac puncture and 5 uniform incisions were made in the rabbit ear. Blood loss was determined by immersing the ear in a saline bath (1 liter) at 37°C for 10 minutes. Red cell count in the bath solution was determined using a Hycel cell counter. The results are expressed as RBCs x 10^9/liter. Data represents a mean ± S.E. N = 5 for saline group. N = 4 for protamine (3 mg/kg i.v.) group. N = 3 for protamine (2 mg/kg i.v.) group.

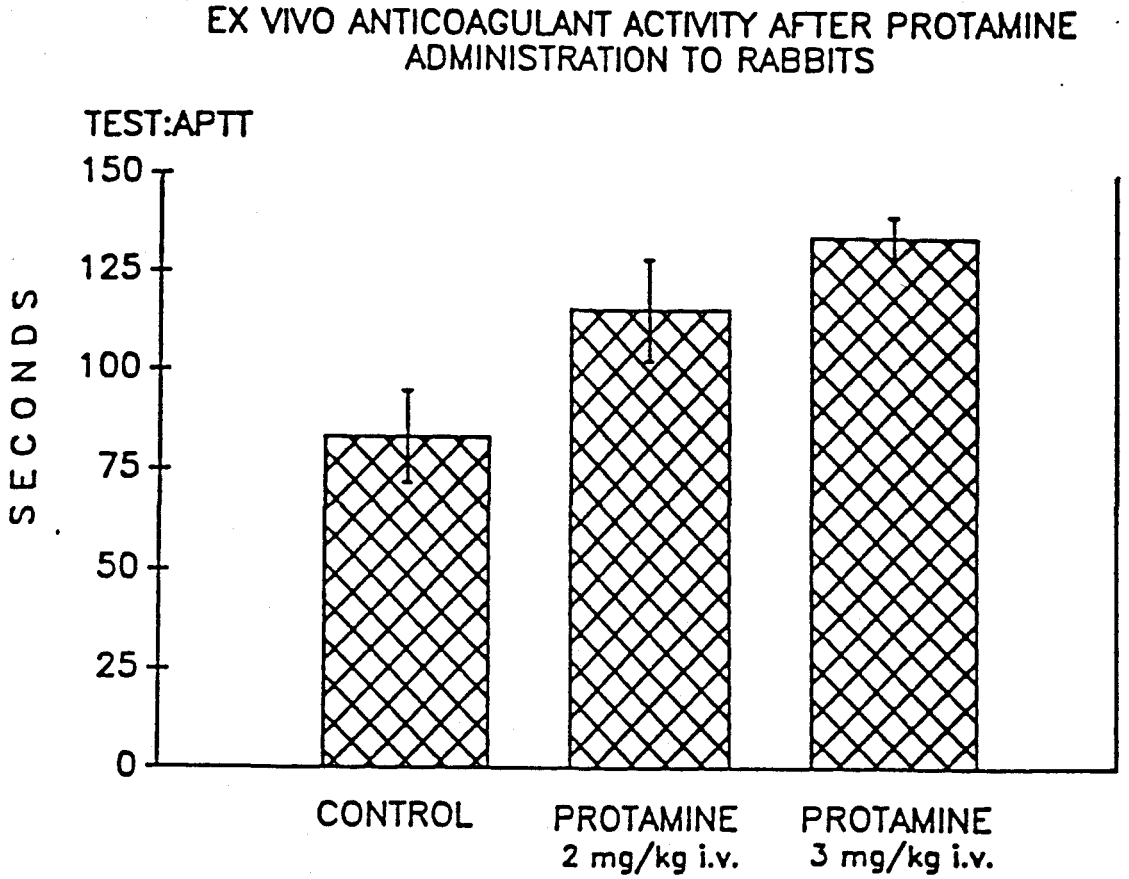


Figure 33. Individual groups of rabbits were administered protamine (2 or 3 mg/kg i.v.) or an equivalent volume of saline. Each drug was allowed to circulate for 10 minutes at which time blood was drawn by cardiac puncture. Anticoagulant activity was determined by the APTT. Data represents a mean ± S.E. N = 5 for control group. N = 4 for protamine (3 mg/kg i.v.) group. N = 3 for protamine (2 mg/kg i.v.) group. Statistical analysis was performed by a one way ANOVA: $F(2,9) = 4.628$, $p > .05$, N.S.

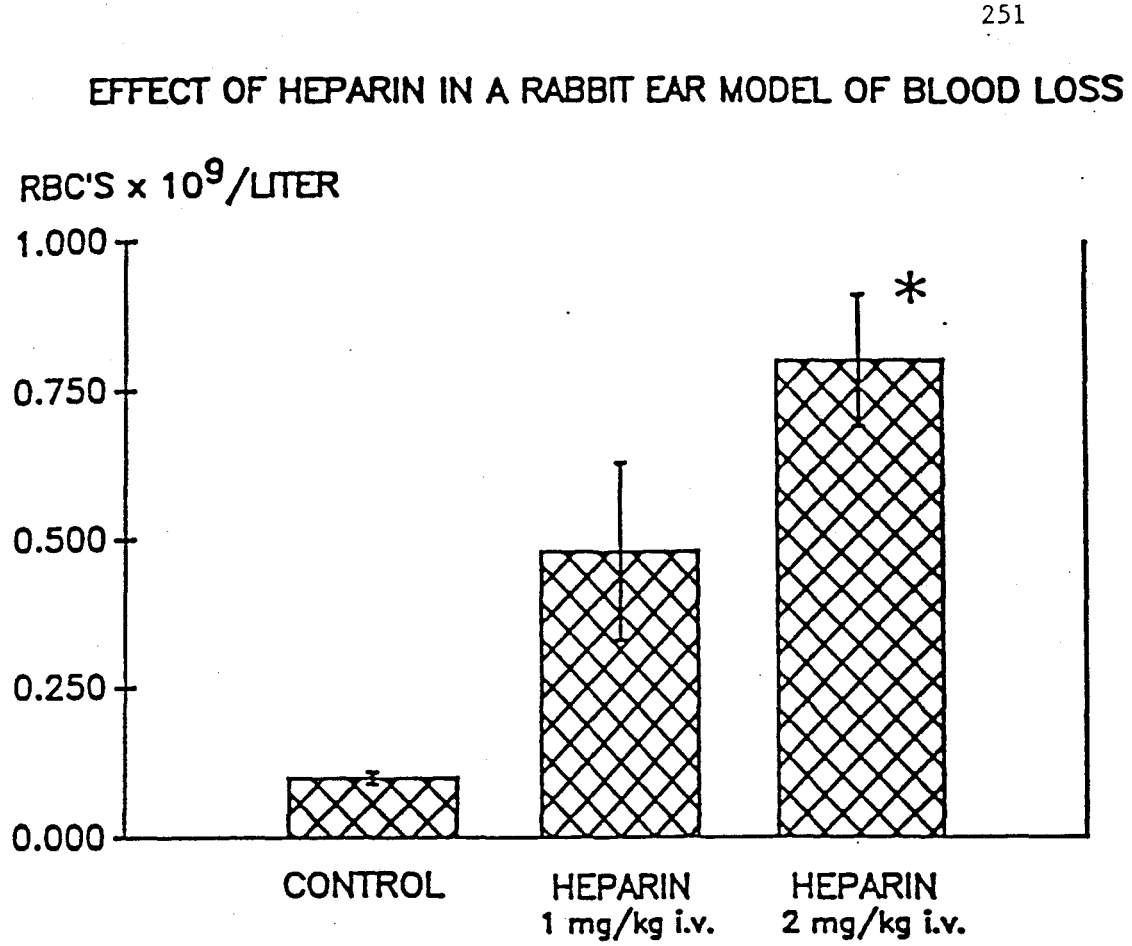


Figure 34. Individual groups of rabbits were administered heparin (1 or 2 mg/kg i.v.) or an equivalent volume of saline which was allowed to circulate for 10 minutes. At this time, blood was drawn by cardiac puncture and 5 uniform incisions were made in the rabbit ear. Blood loss was determined by immersing the ear in a saline bath (1 liter) at 37°C for 10 minutes. Red cell count in the bath solution was determined using a Hycel cell counter. The results were expressed as RBCs x 10^9/liter. Data represents a mean ± S.E. N = 5 for saline group. N = 6 for heparin (1 mg/kg i.v.) group. N = 14 for heparin (2 mg/kg i.v.) group. Statistical analysis was performed by a one way ANOVA: $F(2,22) = 8.473$, $p < .05$ followed by a Newman-Keuls multiple comparison test. * represents a significant difference from the control group.

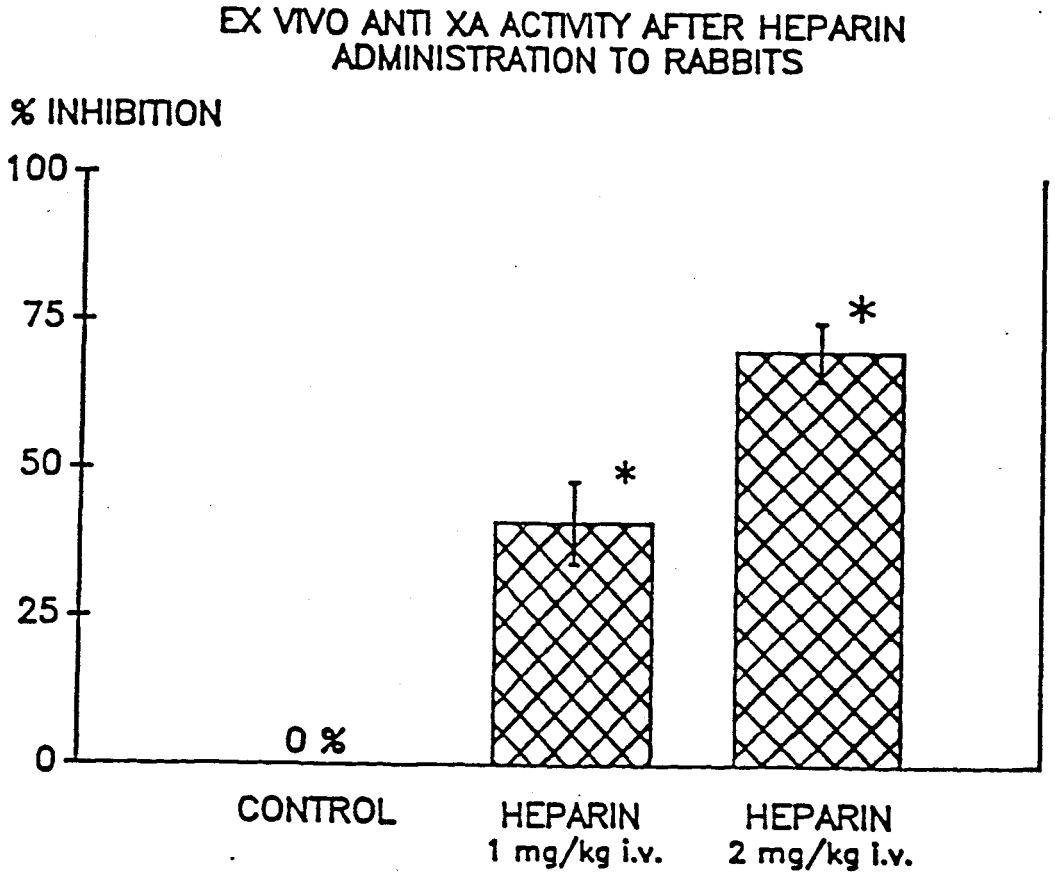


Figure 35. Individual groups of rabbits were administered heparin (1 mg/kg i.v.) or heparin (2 mg/kg i.v.) or an equivalent volume of saline. The drugs were allowed to circulate for 10 minutes at which time blood was drawn by cardiac puncture. Antiprotease activity was determined by an amidolytic anti Xa assay. Data represents a mean ± S.E. N = 5 for saline group. N = 6 for heparin (1 mg/kg i.v.) group. N = 14 for heparin (2 mg/kg i.v.) group. Statistical analysis was performed by a one way ANOVA: $F(2,22) = 32.433$, $p < .05$ followed by a Newman-Keuls multiple comparison test. * represents a significant difference from the control group.

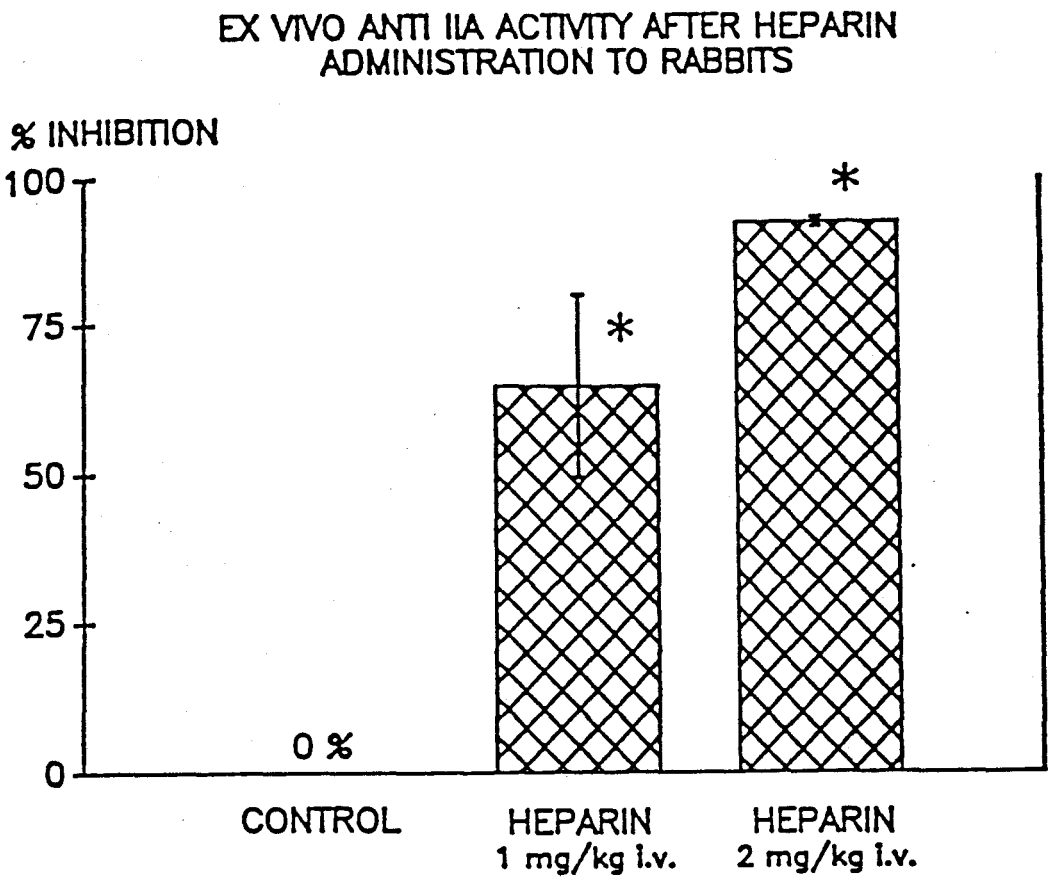


Figure 36. Individual groups of rabbits were administered either heparin (1 or 2 mg/kg i.v.) or an equivalent volume of saline. The drugs were allowed to circulate for 10 minutes at which time blood was drawn by cardiac puncture. Antiprotease activity was determined by an amidolytic anti IIa assay. Data represents a mean ± S.E. N = 5 for saline group. N = 6 for heparin (1 mg/kg i.v.) group. N = 14 for heparin (2 mg/kg i.v.) group. Statistical analysis was performed by a one way ANOVA: $F(2,22) = 34.378$, $p < .05$ followed by a Newman-Keuls multiple comparison test. * represents a significant difference from control group.

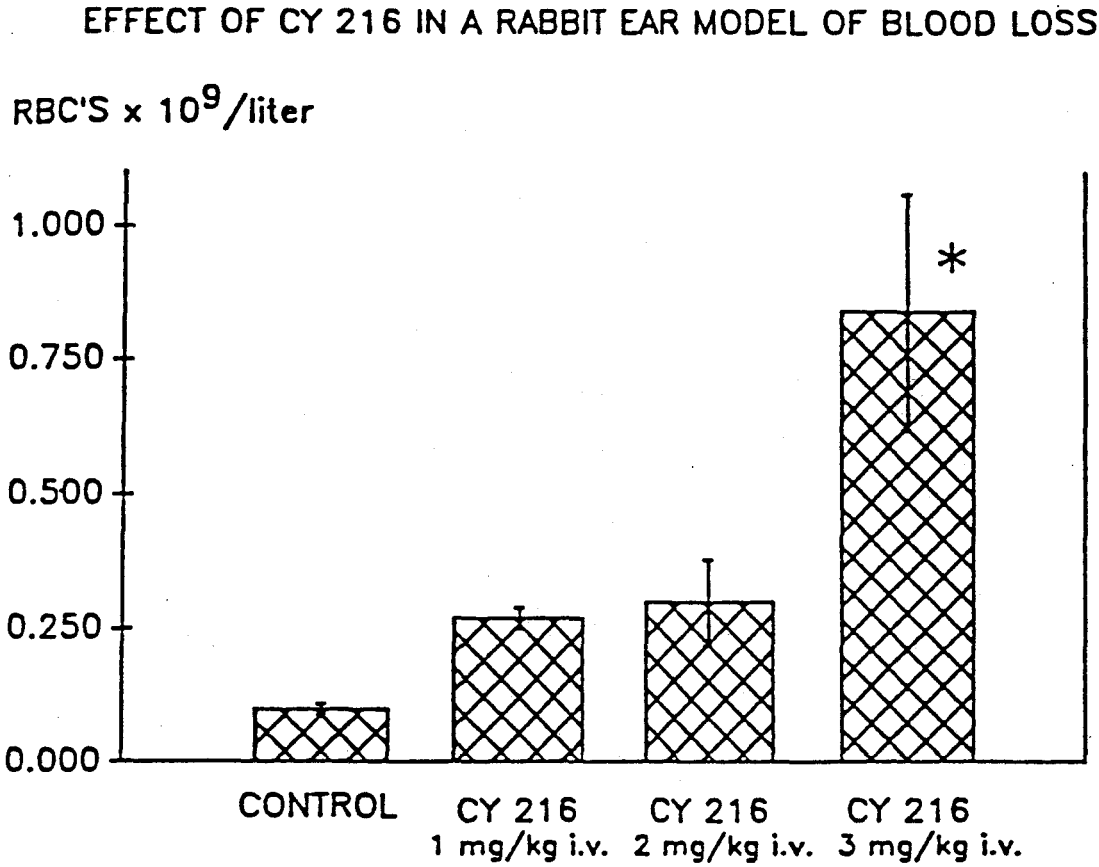


Figure 37. Individual groups of rabbits were administered, CY 216 (1, 2 or 3 mg/kg i.v.) or an equivalent volume of saline which was allowed to circulate for 10 minutes. At this time, blood was drawn by cardiac puncture and 5 uniform incisions were made in the ear. Blood loss was determined by immersing the ear in a saline bath (1 liter) at 37°C for 10 minutes. Red cell count in the bath solution was determined using a Hycel cell counter. The results were expressed as RBCs x 10^9/liter. Data represents a mean ± S.E. N = 14 for CY 216 (2 mg/kg i.v.) group. N = 5 for all other groups. Statistical analysis was performed by a one way ANOVA: $F(3,25) = 8.505$, $p < .05$ and followed by a Newman-Keuls multiple comparison test. * represents significant difference from control group.

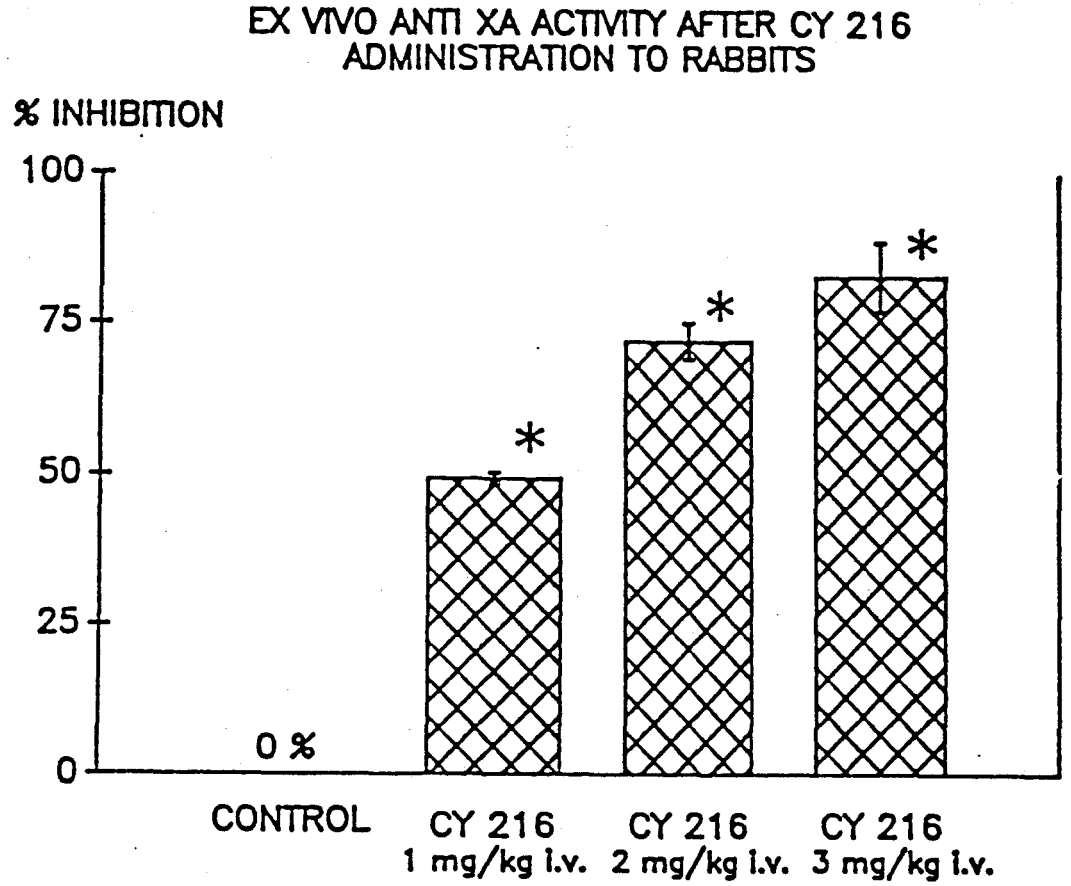


Figure 38. Individual groups of rabbits were administered CY 216 (1 mg/kg i.v.), CY 216 (2 mg/kg i.v.) or CY 216 (3 mg/kg i.v.) or an equivalent volume of saline. The drugs were allowed to circulate for 10 minutes at which time blood was drawn by cardiac puncture. Antiprotease activity was determined by an amidolytic anti Xa assay. Data represents a mean ± S.E. N = 14 for CY 216 (2 mg/kg i.v.) group. N = 5 for all other groups. Statistical analysis was performed by a one way ANOVA: $F(3,25) = 16.450$, $p < .05$ and followed by a Newman-Keuls multiple comparison test. * represents a significant difference from the control group.

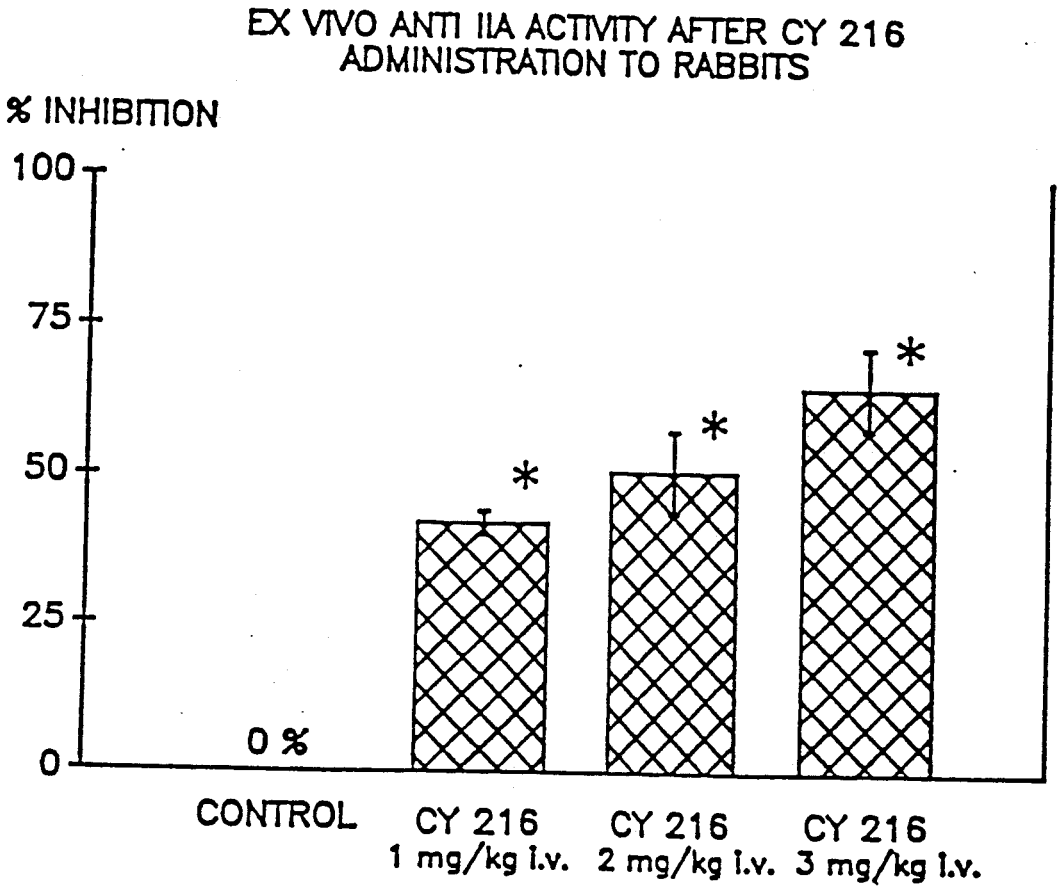


Figure 39. Individual groups of rabbits were administered CY 216 (1, 2 or 3 mg/kg i.v.) or an equivalent volume of saline. The drugs were allowed to circulate for 10 minutes at which time blood was drawn by cardiac puncture. Antiprotease activity was determined by an amidolytic anti IIa assay. Data represents a mean ± S.E. N = 14 for CY 216 (2 mg/kg i.v.) group. N = 5 for all other groups. Statistical analysis was performed by a one way ANOVA: $F(3,25) = 78.307$, $p < .05$ and followed by a Newman-Keuls multiple comparison test. * represents a significant difference from the control group.

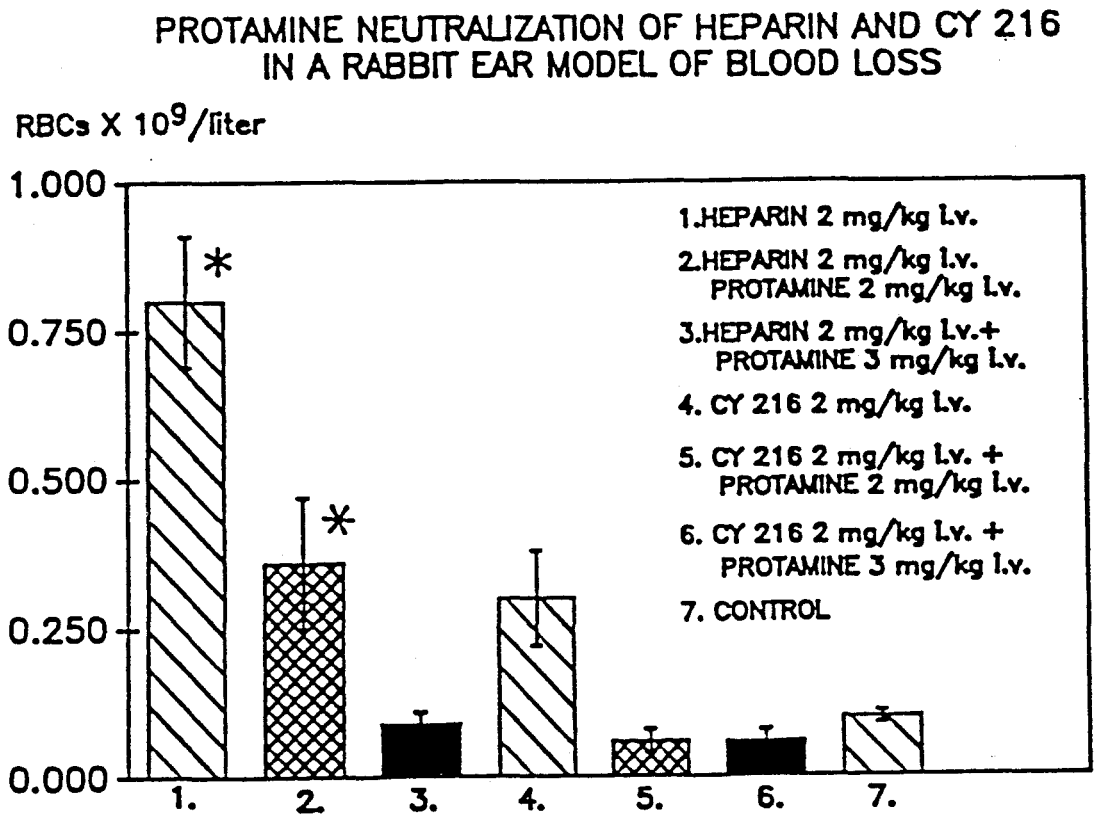


Figure 40. Seven groups of rabbits were administered heparin (2 mg/kg i.v.) or CY 216 (2 mg/kg i.v.) or an equivalent volume of saline via the right ear vein. Each drug was allowed to circulate for 10 minutes at which time blood was drawn by cardiac puncture and blood loss was determined. Four groups received protamine (2 or 3 mg/kg i.v.) via the left ear vein, 5 minutes after the administration of heparin (2 mg/kg i.v.) or CY 216 (2 mg/kg i.v.) and blood was drawn by cardiac puncture. Five uniform incisions were made in the ear. Blood loss was determined by immersing the ear in a saline bath (1 liter) at 37°C for 10 minutes. Red cell count in the bath solution was determined using a Hycel sell counter. The results were expressed as RBCs x 10^9/liter. Data represents a mean ± S.E. N = 6 for heparin (2 mg/kg i.v.) or CY 216 (2 mg/kg i.v.) and protamine (2 mg/kg i.v.). N = 5 for all other groups. Statistical analysis was performed by a two way ANOVA:

Factor A (drug treatment): $F(2,36) = 17.494$ $p < .05$
Factor B (protamine dose): $F(2,36) = 12.806$ $p < .05$
Factor A x B: $F(4,36) = 6.318$ $p < .05$

followed by a Newman-Keuls multiple comparison test. * represents a significant difference from the control group.

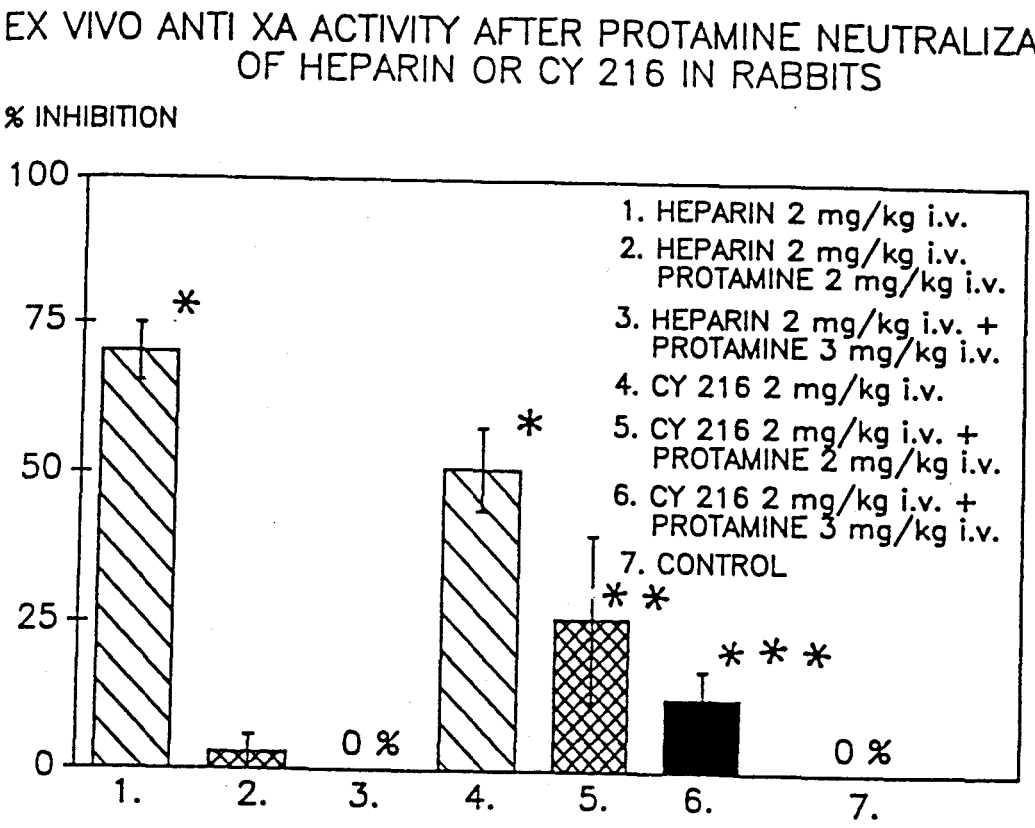


Figure 41. Individual groups of rabbits were administered heparin (2 mg/kg i.v.) or CY 216 (2 mg/kg i.v.) or an equivalent volume of saline via the right ear vein. Each drug was allowed to circulate for 10 minutes at which time blood was drawn by cardiac puncture and blood loss was determined. Four groups received protamine (2 or 3 mg/kg i.v.) via the left ear vein, 5 minutes after the administration of heparin (2 mg/kg i.v.) or CY 216 (2 mg/kg i.v.) and blood was drawn by cardiac puncture. Antiprotease activity was determined by an amidolytic anti Xa assay. Data represents a mean ± S.E. N = 6 for heparin or CY 216 (2 mg/kg i.v.) and protamine (2 mg/kg i.v.) group. N = 5 for all other groups. Statistical analysis was performed by a two way ANOVA:

Factor A (drug treatment): $F(2,36) = 29.090$ $p < .05$
Factor B (protamine dose): $F(2,36) = 62.246$ $p < .05$
Factor A x B: $F(4,36) = 18.790$ $p < .05$

followed by a Newman-Keuls multiple comparison test.
* represents a significant difference from control group.
** represents a significant difference from heparin (2 mg/kg i.v.) and protamine (2 mg/kg i.v.) group.
*** represents a significant difference from heparin (2 mg/kg i.v.) and protamine (3 mg/kg i.v.) group.

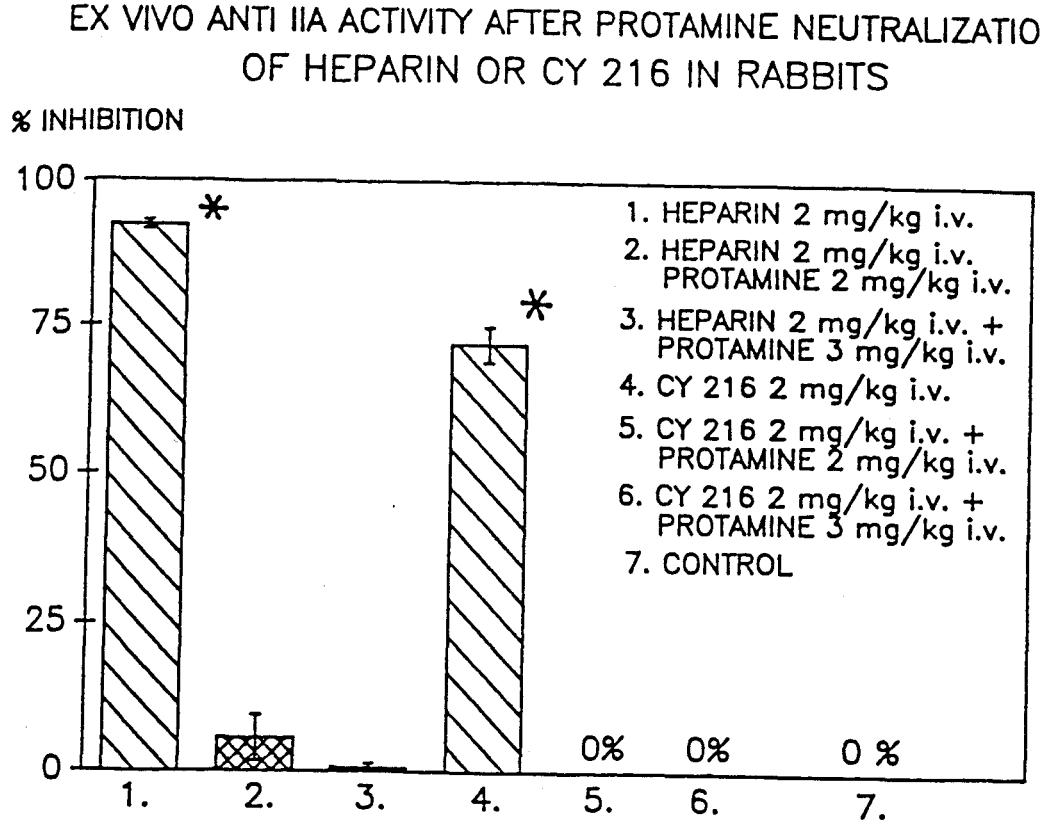


Figure 42. Individual groups of rabbits were administered an equivalent amount of saline, heparin (2 mg/kg i.v.) or CY 216 (2 mg/kg i.v.) via the right ear vein. Each drug was allowed to circulate for 10 minutes at which time blood was drawn by cardiac puncture and blood loss was determined. Four groups received protamine (2 or 3 mg/kg i.v.) via the left ear vein, 5 minutes after the administration of heparin (2 mg/kg i.v.) or CY 216 (2 mg/kg i.v.) and blood was drawn by cardiac puncture. Antiprotease activity was determined by a amidolytic anti IIa assay. Data represents a mean ± S.E. N = 6 for heparin or CY 216 (2 mg/kg i.v.) and protamine (1 mg/kg i.v.) group. N = 5 for all other groups. Statistical analysis was performed by a two way ANOVA:

Factor A (drug treatment): $F(2,36) = 71.372$ $p < .05$
Factor B (protamine dose): $F(2,36) = 213.532$ $p < .05$
Factor A x B: $F(4,36) = 55.335$ $p < .05$

followed by a Newman-Keuls multiple comparison test.
* represents significant difference from the control group.

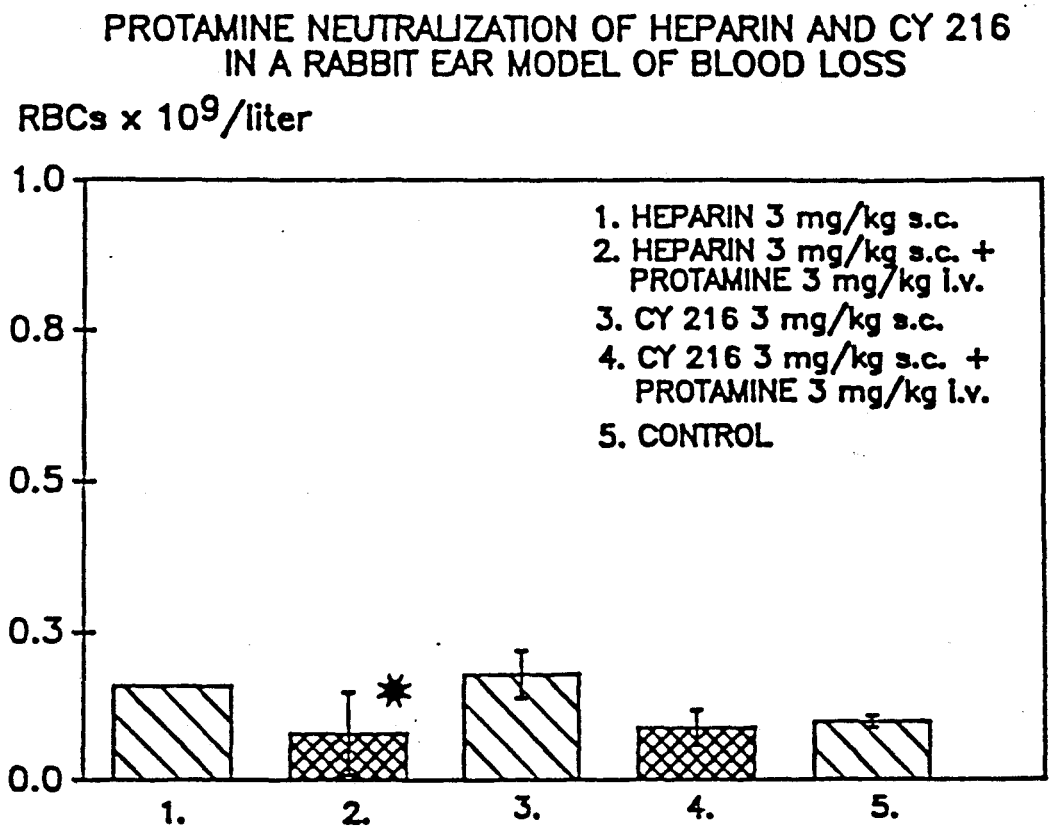


Figure 43. Individual groups consisting of five rabbits each were administered heparin or CY 216 (3 mg/kg s.c.) or an equivalent volume of saline via the right ear vein. The drugs were allowed to circulate for 120 minutes at which time blood was drawn by cardiac puncture. Blood loss was determined by immersing the ear in a saline bath (1 liter) at 37°C for 10 minutes. Red cell count in the bath solution was determined using a Hycel cell counter. The results were expressed as RBCs x 10^9/liter. Data represents a mean ± S.E. Statistical analysis was performed by a two way ANOVA:

Factor A (drug treatment): $F(2,24) = .601$ $p > .50$ N.S.
Factor B (protamine dose): $F(1,24) = 18.175$ $p < .05$
Factor A x B: $F(2,24) = 4.239$ $p > .05$ N.S.

followed by a Newman Keuls multiple comparison test. * represents a significant difference from the CY 216 (3 mg/kg s.c.) and protamine (3 mg/kg i.v.) group.

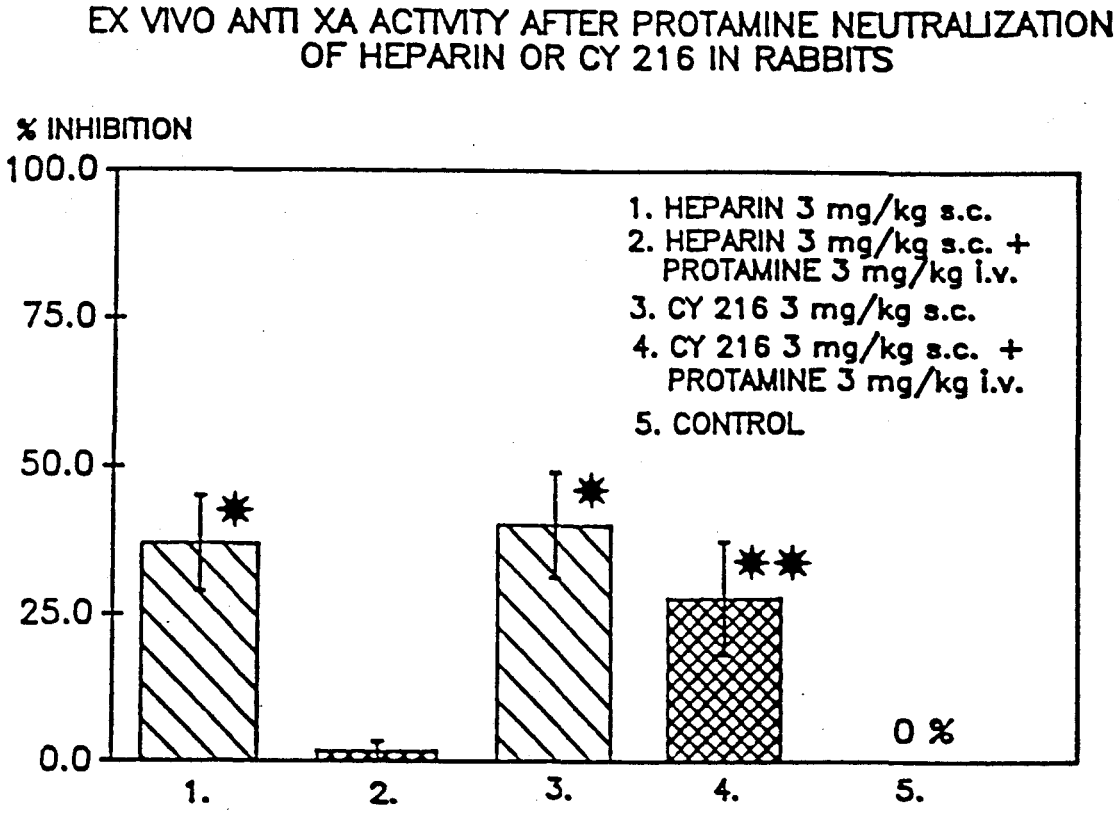


Figure 44. Individual groups consisting of five rabbits each were administered heparin (3 mg/kg s.c.) or CY 216 (3 mg/kg s.c.) Each agent was allowed to circulate for 120 minutes at which time blood was drawn by cardiac puncture. Two groups received protamine sulfate (3 mg/kg i.v.) 120 minutes after the administration of heparin (3 mg/kg s.c.) or CY 216 (3 mg/kg s.c.). Protamine was allowed to circulate for 5 minutes at which time blood was drawn by cardiac puncture. Antiprotease activity was determined by an amidolytic anti Xa assay. Data represents a mean ± S.E. Statistical analysis was performed by a two way ANOVA:

Factor A (drug treatment): $F(2,24) = 26.802$ $p < .05$
Factor B (protamine dose): $F(1,24) = 17.158$ $p < .05$
Factor A x B: $F(2,24) = 8.056$ $p < .05$

followed by a Newman-Keuls multiple comparison test.
* represents a significant difference from the control group.
** represents a significant difference from the heparin (3 mg/kg s.c.) and protamine (3 mg/kg i.v.) group.

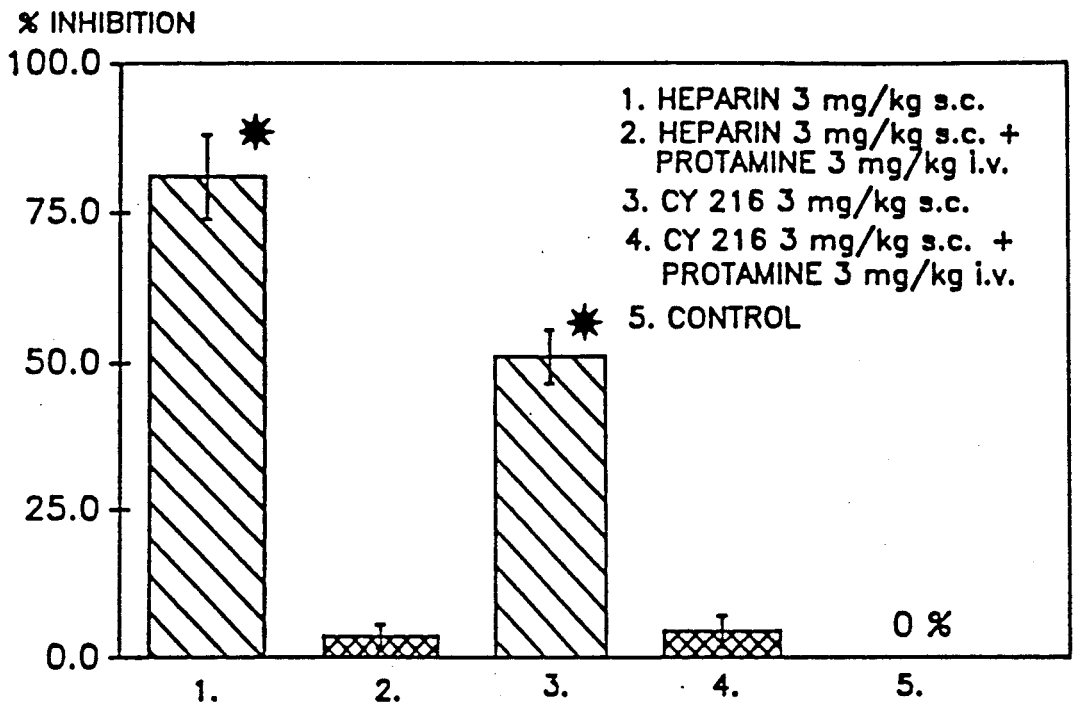


Figure 45. Individual groups consisting of five rabbits each were administered heparin (3 mg/kg s.c.) or CY 216 (3 mg/kg s.c.). The drugs were allowed to circulate for 120 minutes at which time blood was drawn by cardiac puncture. Two groups received protamine sulfate (3 mg/kg i.v.) 120 minutes after the administration of heparin (3 mg/kg s.c.) or CY 216 (3 mg/kg s.c.). Protamine was allowed to circulate for 5 minutes at which time blood was drawn by cardiac puncture. Antiprotease activity was determined by an amidolytic anti IIa assay. Data represents a mean ± S.E. Statistical analysis was performed by a two way ANOVA:

Factor A (drug treatment): $F(2,24) = 198.315$ $p < .05$
Factor B (protamine dose): $F(1,24) = 488.486$ $p < .05$
Factor A x B: $F(2,24) = 162.957$ $p < .05$

followed by a Newman-Keuls multiple comparison test. * represents a significant difference from the control group.

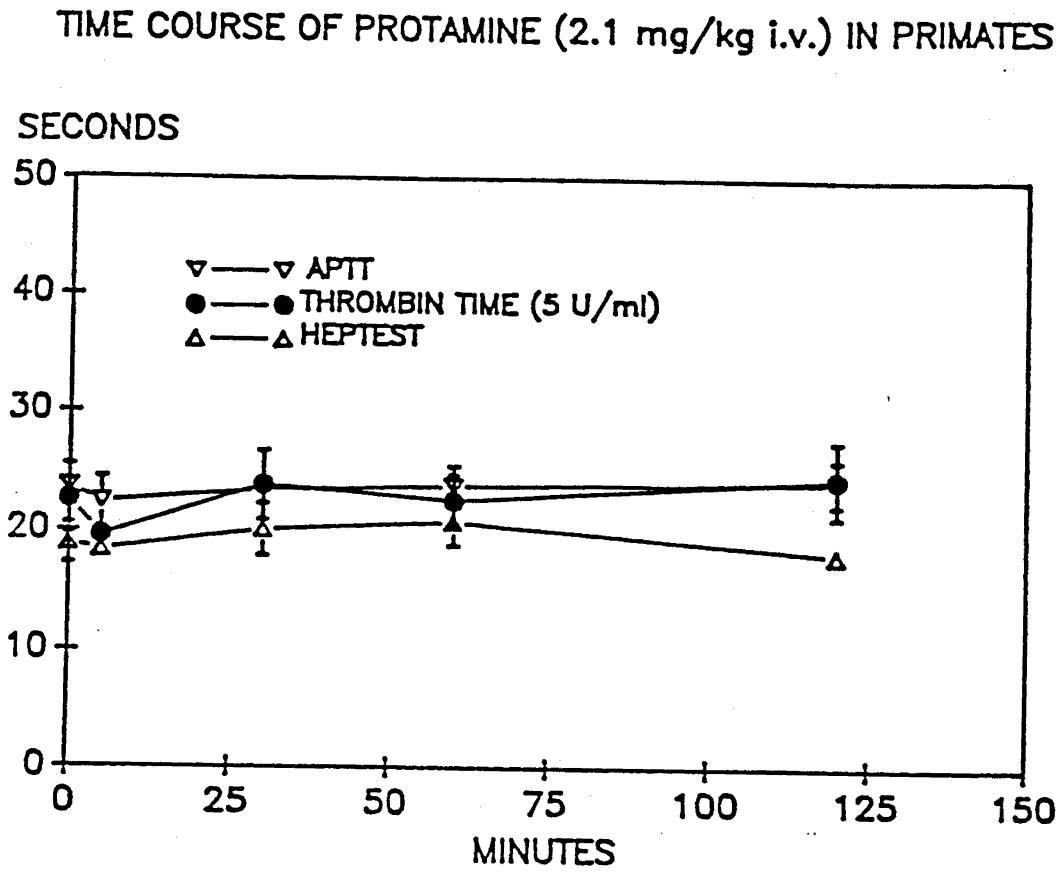


Figure 46. A group of five primates was administered protamine (2.1 mg/kg i.v.). Blood samples were drawn at 0, 5, 30, 60 and 120 minutes. Anticoagulant and antiprotease activity was determined using the APTT, thrombin time and Heptest^{R}. Each data point represents a mean ± S.E.

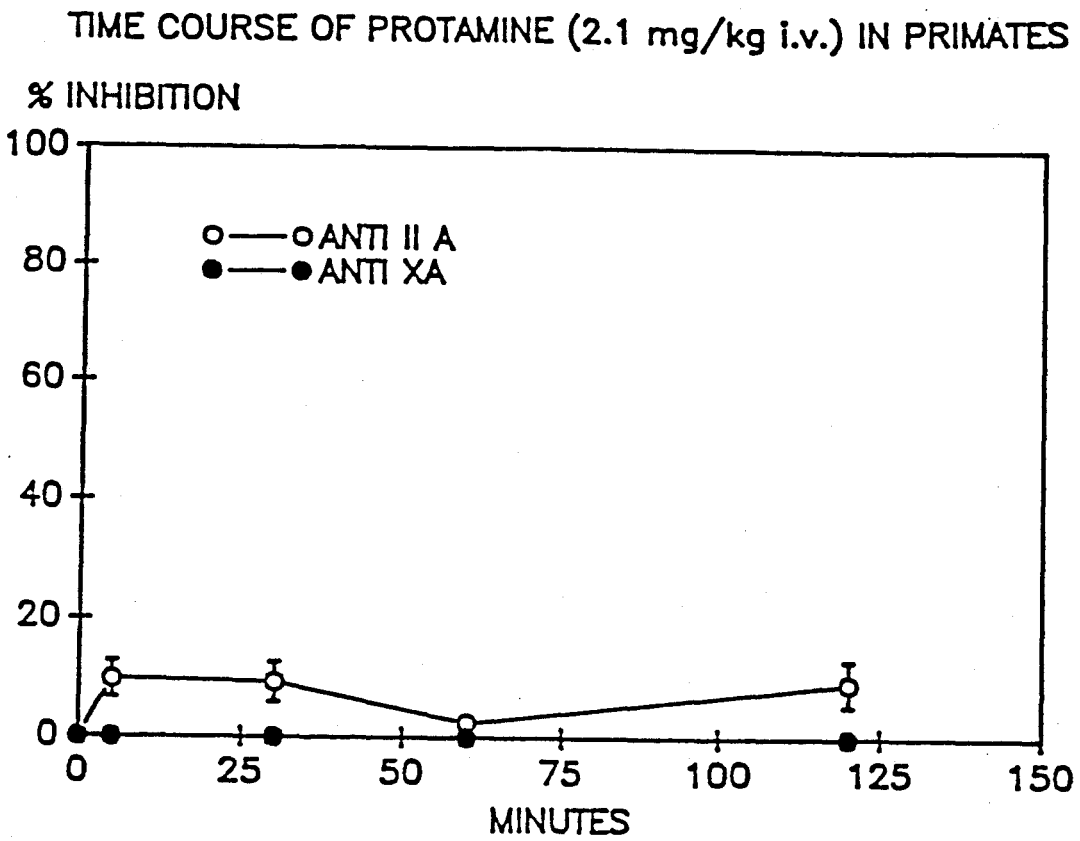


Figure 47. A group of 5 primates was administered protamine (2.1 mg/kg i.v.). Blood samples were drawn at 0, 5, 30, 60 and 120 minutes. Antiprotease activities were determined using amidolytic anti Xa and amidolytic anti IIa assays. Each data point represents a mean ± S.E. Statistical anlysis was performed by a two way ANOVA:

Factor A (time): $F(4,40) = 3.160$ $p>.05$ N.S.
Factor B (test): $F(1,40) = 26.873$ $p<.05$
Factor A x B: $F(4,40) = 3.160$ $p>.05$ N.S.

followed by a Newman-Keuls multiple comparison test. * represents a significant difference from the control group.

TIME COURSE OF PROTAMINE SULFATE (2.1 mg/kg i.v.) IN PRIMATES AS DETERMINED BY AN INDIRECT METHOD

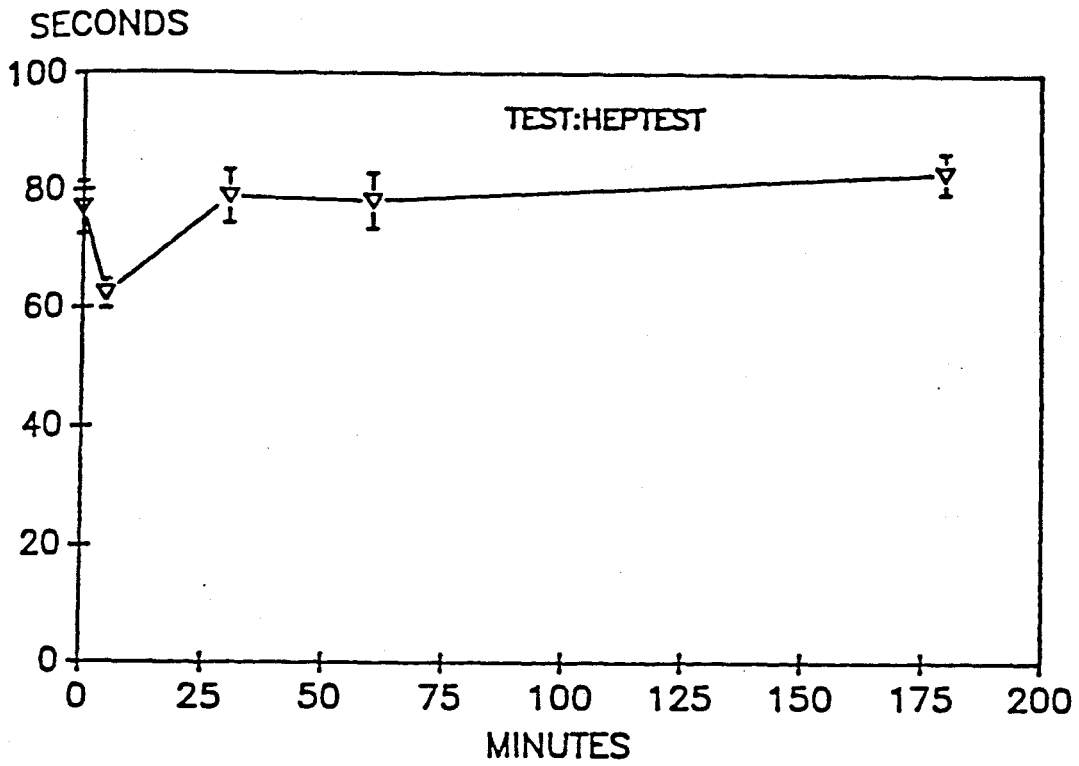


Figure 48. A group of four primates was administered protamine (2.1 mg/kg i.v.). Blood samples were drawn at 0, 5, 30, 60 and 180 minutes. The plasma samples were centrifuged and frozen at -70°C. The pharmacodynamics of protamine were assayed indirectly by supplementing 0.63 ml of plasma obtained after protamine administration to the primates with 70 μl of heparin 25 μg/ml. The final concentration of heparin in the plasma sample was 2.5 μg/ml. Heptest^{R} was performed. Data represents a mean ± S.E. Statistical analysis was performed by a two way ANOVA:

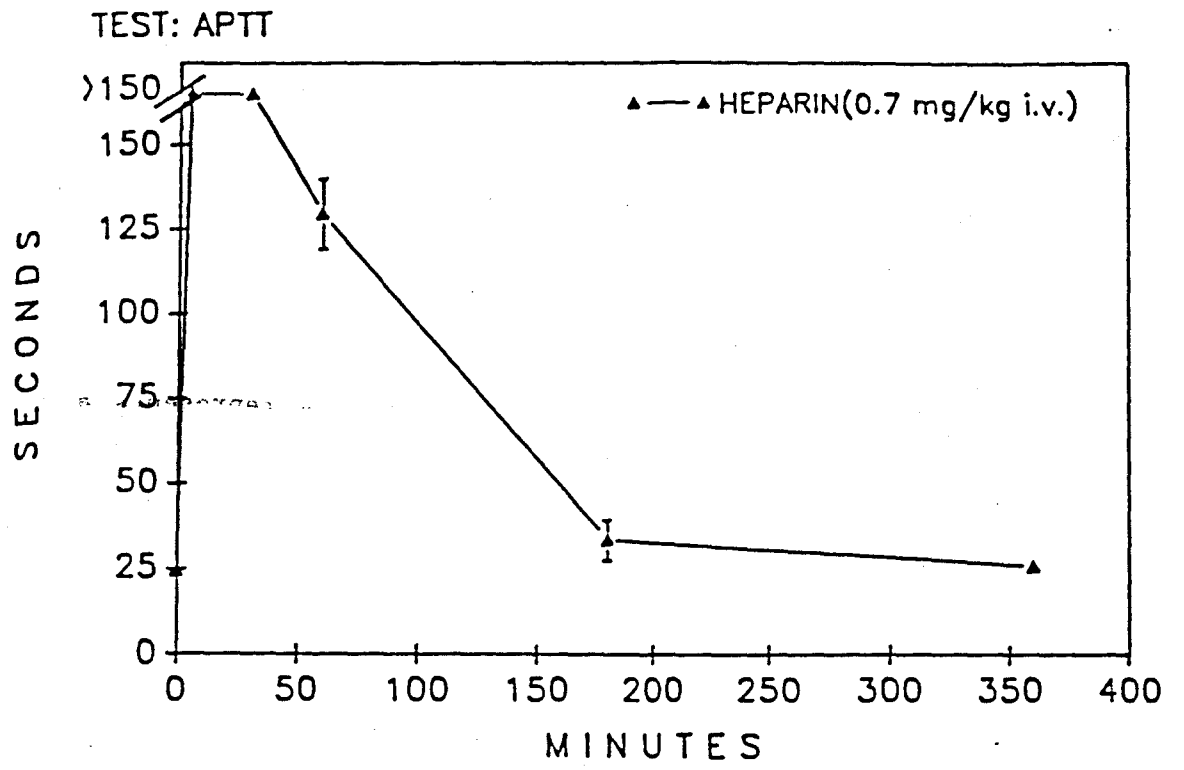
Factor A (time): $F(4,40) = 1.728$ $p>.50$ N.S.
Factor B (protamine dose): $F(1,40) = .167$ $p>.50$ N.S.
Factor (A x B): $F(4,40) = 1.728$ $p>.50$ N.S.

Figure 49. Four groups of primates were included in this study. The first group (N = 4) was administered heparin (0.7 mg/kg i.v.) (upper panel). The other three groups (N = 5) received heparin (0.7 mg/kg i.v.) followed by protamine sulfate (0.7, 1.4, or 2.1 mg/kg i.v.), five minutes after heparin administration (lower panel). Blood samples from each of these 4 groups were drawn at 0, 5, 30, 60, 180 and 360 minutes. An additional blood sample was obtained at 15 minutes from primates which were treated with protamine at the 5 minute time period. Anticoagulant activity was determined using the APTT. Each data point represents a mean ± S.E. Statistical analysis was performed by a two way ANOVA:

Factor A (time): $F(3,40) = 3.398$ $p>.05$ N.S.
Factor B (protamine dose): $F(2,40) = 25.634$ $p<.05$
Factor A x B: $F(6,40) = 1.300$ $p>.20$ N.S.

followed by a Newman-Keuls multiple comparison test. * represents a significant difference from heparin (0.7 mg/kg i.v.) and protamine (1.4 mg/kg i.v.) group and heparin (0.7 mg/kg i.v.) and protamine (2.1 mg/kg i.v.) group.

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF HEPARIN IN PRIMATES



WITH PROTAMINE SULFATE

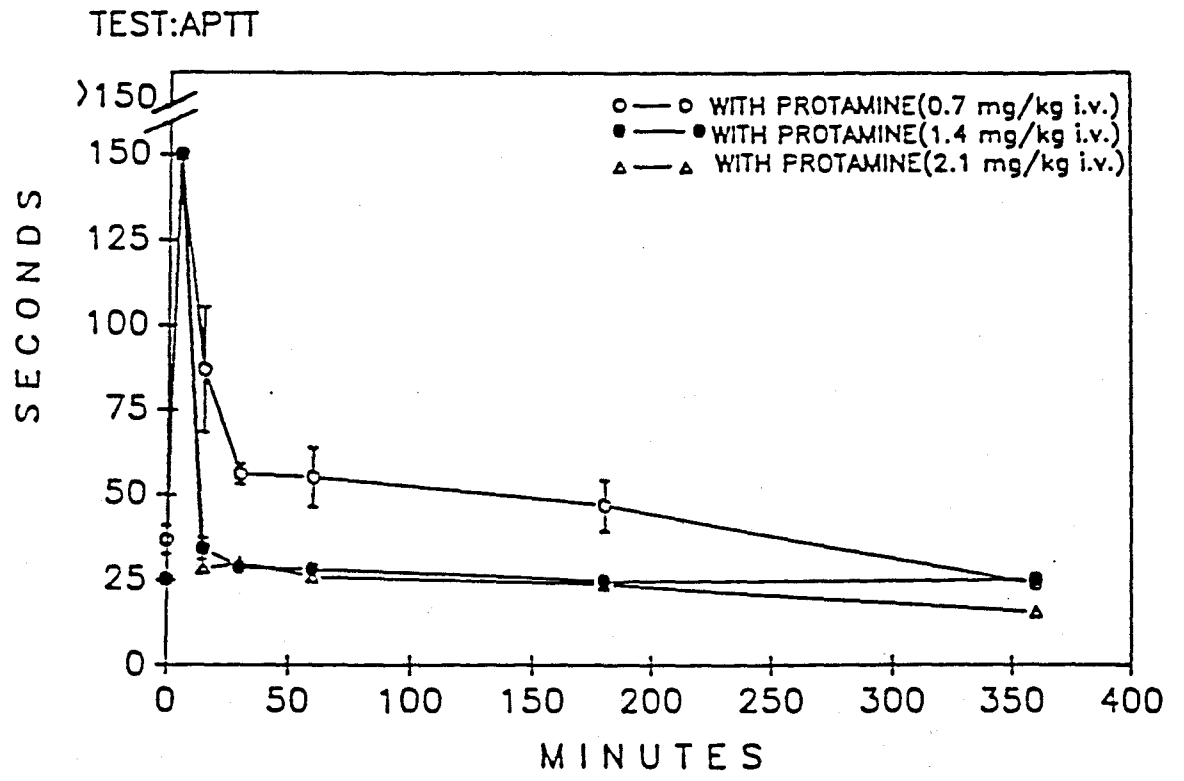


Figure 50. Four groups of primates were included in this study. The first group (N = 4) was administered heparin (0.7 mg/kg i.v.), (upper panel). The other three groups (N = 5) received heparin (0.7 mg/kg i.v.) followed by protamine sulfate (0.7, 1.4, or 2.1 mg/kg i.v.), five minutes after heparin administration (lower panel). Blood samples from each of these 4 groups were drawn at 0, 5, 30, 60, 180 and 360 minutes. An additional blood sample was obtained at 15 minutes from primates which were treated with protamine at the 5 minute time period. Anticoagulant activity was determined using the thrombin time. Each data point represents a mean ± S.E. Statistical analysis was performed by a two way ANOVA:

Factor A (protamine dose): $F(2,29) = 1.863$ $p>.50$ N.S.

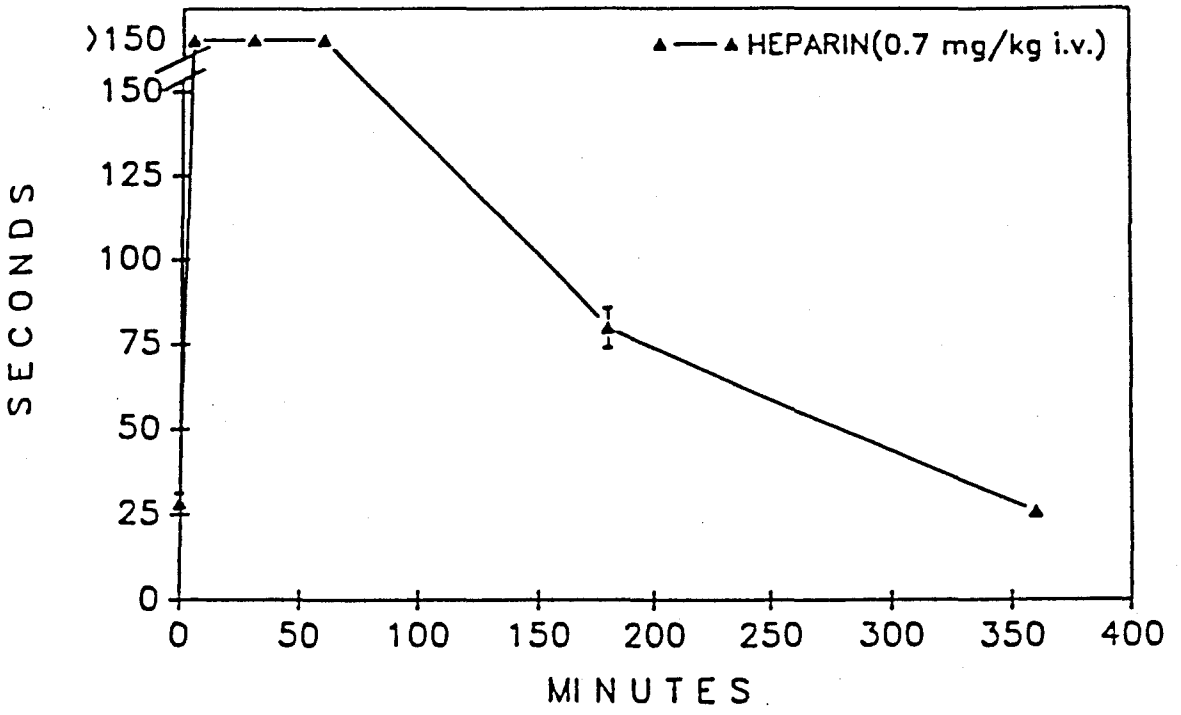
Factor A (time): $F(2,29) = 6.648$ $p<.01$

Factor A x B: $F(4,29) = 3.121$ $p>.05$ N.S.

followed by a Newman-Keuls Multiple comparison test. * represents a significant difference from heparin (0.7 mg/kg i.v.) and protamine (1.4 mg/kg i.v.) group and the heparin (0.7 mg/kg i.v.) and protamine (2.1 mg/kg i.v.) group.

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF HEPARIN IN PRIMATES

TEST: THROMBIN TIME (5 U/ml)



WITH PROTAMINE SULFATE

TEST: THROMBIN TIME (5 U/ml)

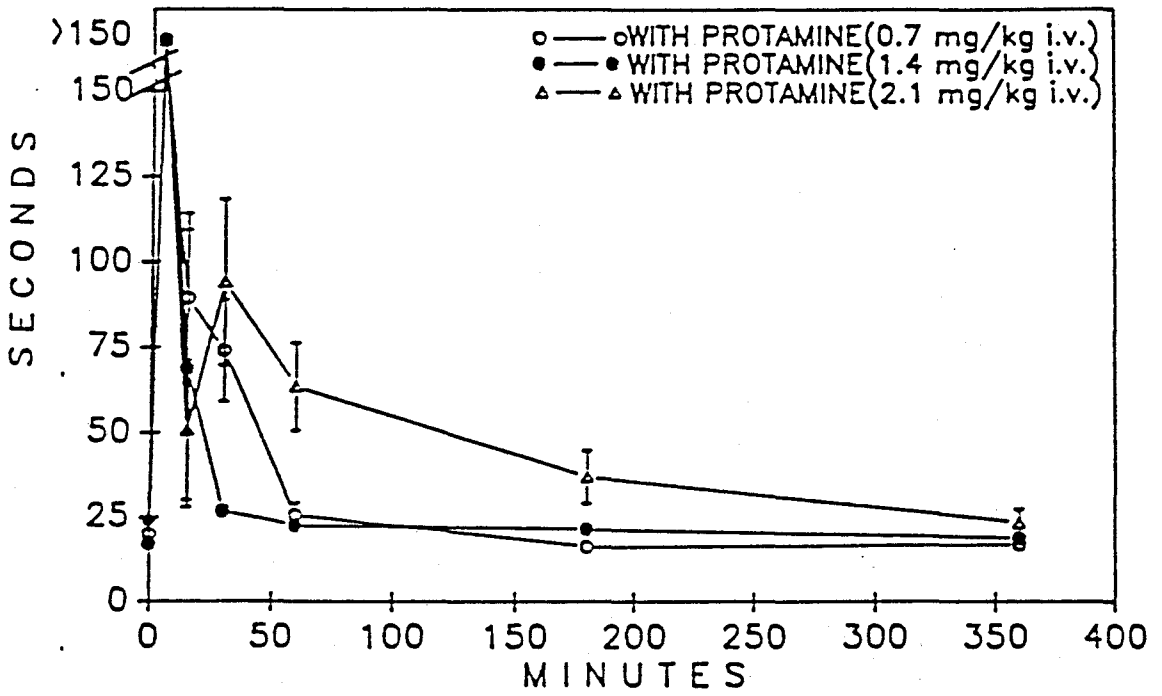
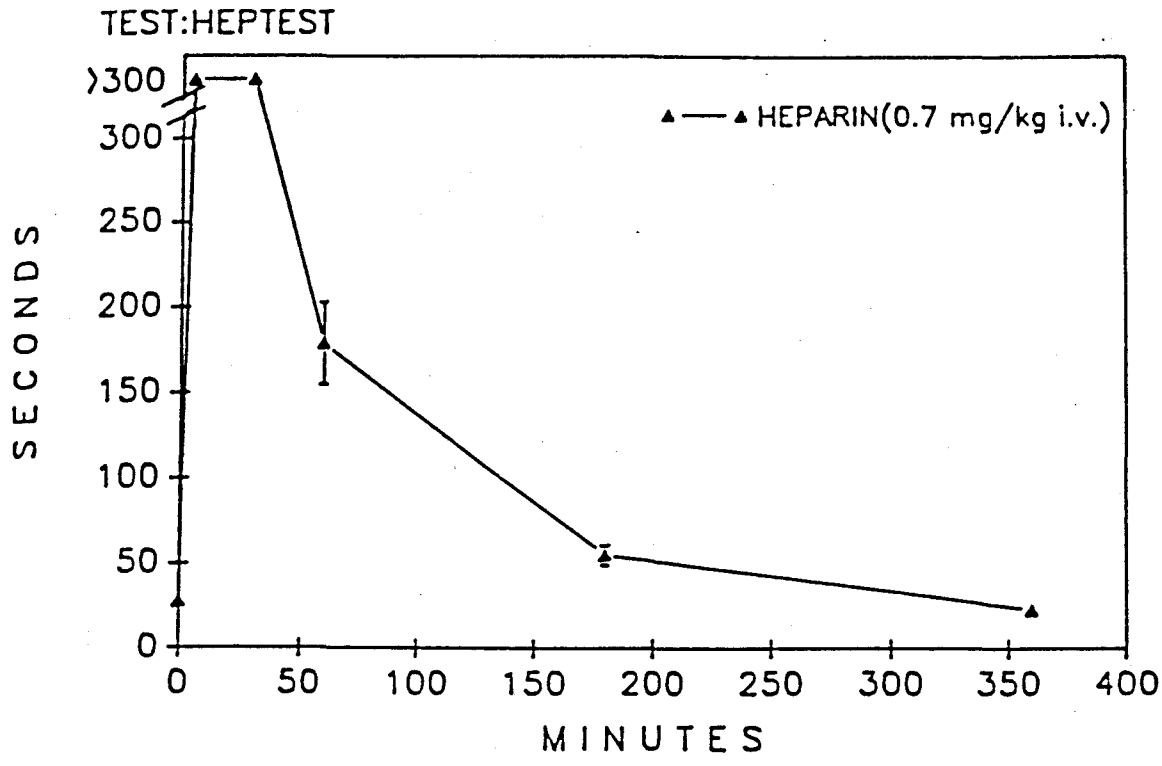


Figure 51. Four groups of primates were included in this study. The first group (N = 4) was administered heparin (0.7 mg/kg i.v.) (upper panel). The other three groups (N = 5) received heparin (0.7 mg/kg i.v.) followed by protamine sulfate (0.7, 1.4, or 2.1 mg/kg i.v.), five minutes after heparin administration (lower panel). Blood samples from each of these 4 groups were drawn at 0, 5, 30, 60, 180 and 360 minutes. An additional blood sample was obtained at 15 minutes from primates which were treated with protamine at the 5 minute time period. Antiprotease activity was determined by the Heptest^{R}. Each data point represents a mean ± S.E.

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF HEPARIN IN PRIMATES



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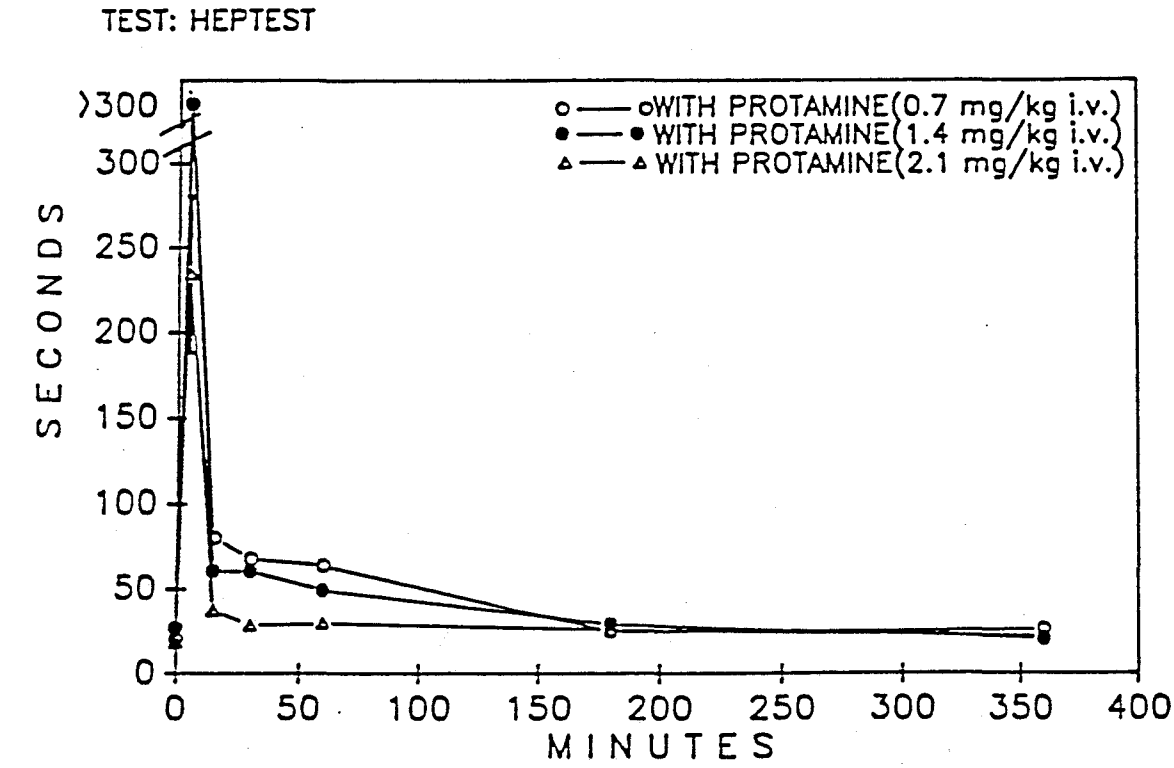
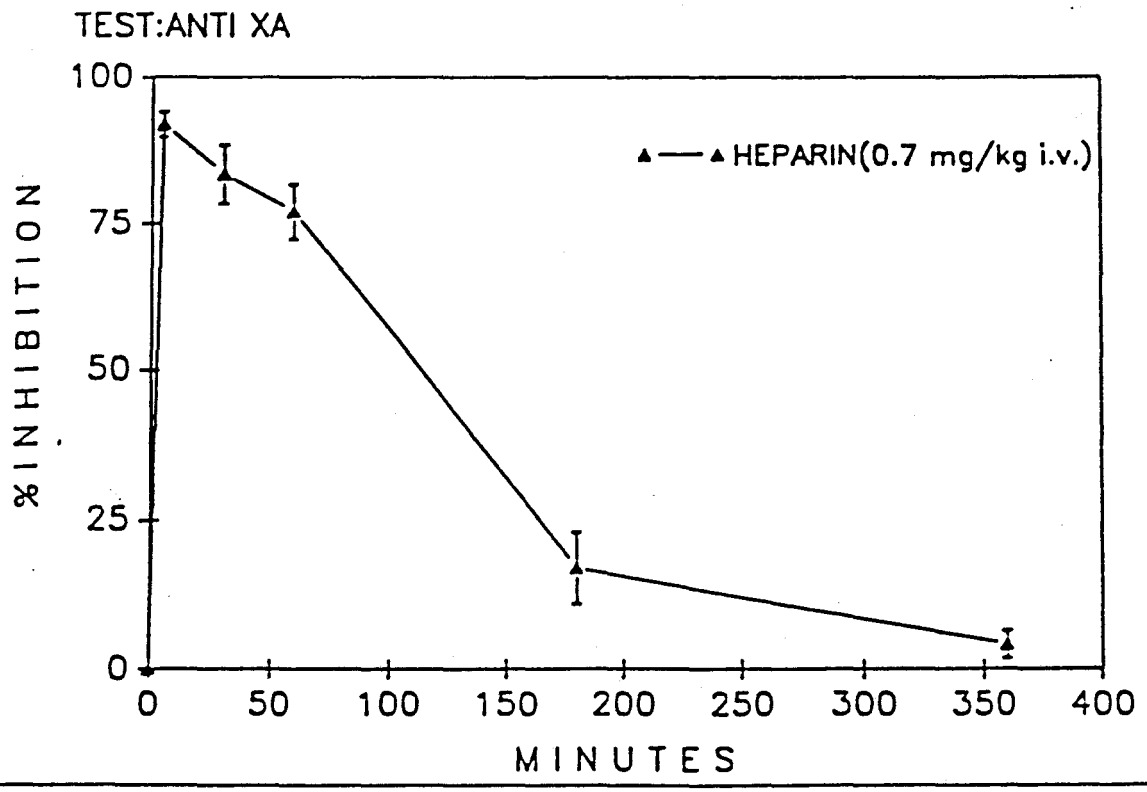


Figure 52. Four groups of primates were included in this study. The first group (N = 4) was administered heparin (0.7 mg/kg i.v.), (upper panel). The other three groups (N = 5) received heparin (0.7 mg/kg i.v.) followed by protamine sulfate (0.7, 1.4, or 2.1 mg/kg i.v.), five minutes after heparin administration (lower panel). Blood samples from each of these 4 groups were drawn at 0, 5, 30, 60, 180 and 360 minutes. An additional blood sample was obtained at 15 minutes from primates which were treated with protamine at the 5 minutes time period. Antiprotease activity was determined using an amidolytic anti Xa assay. Each data point represents a mean ± S.E.

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY
OF HEPARIN IN PRIMATES



WITH PROTAMINE SULFATE

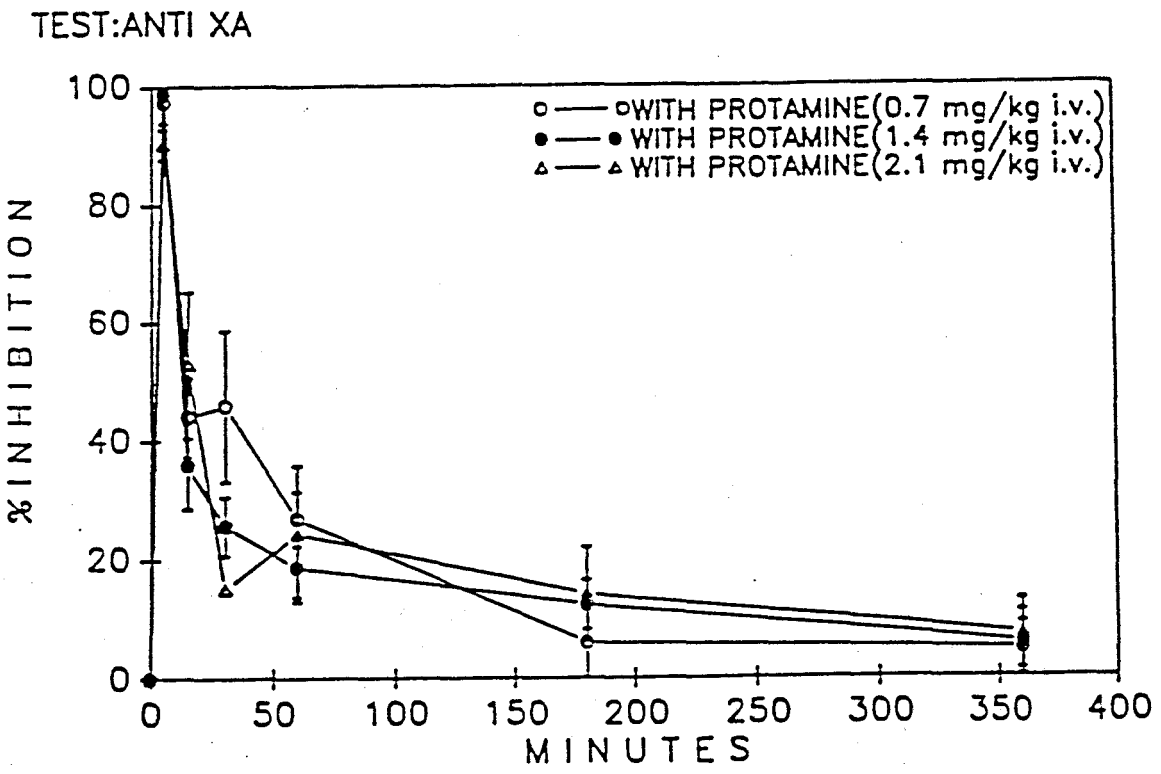
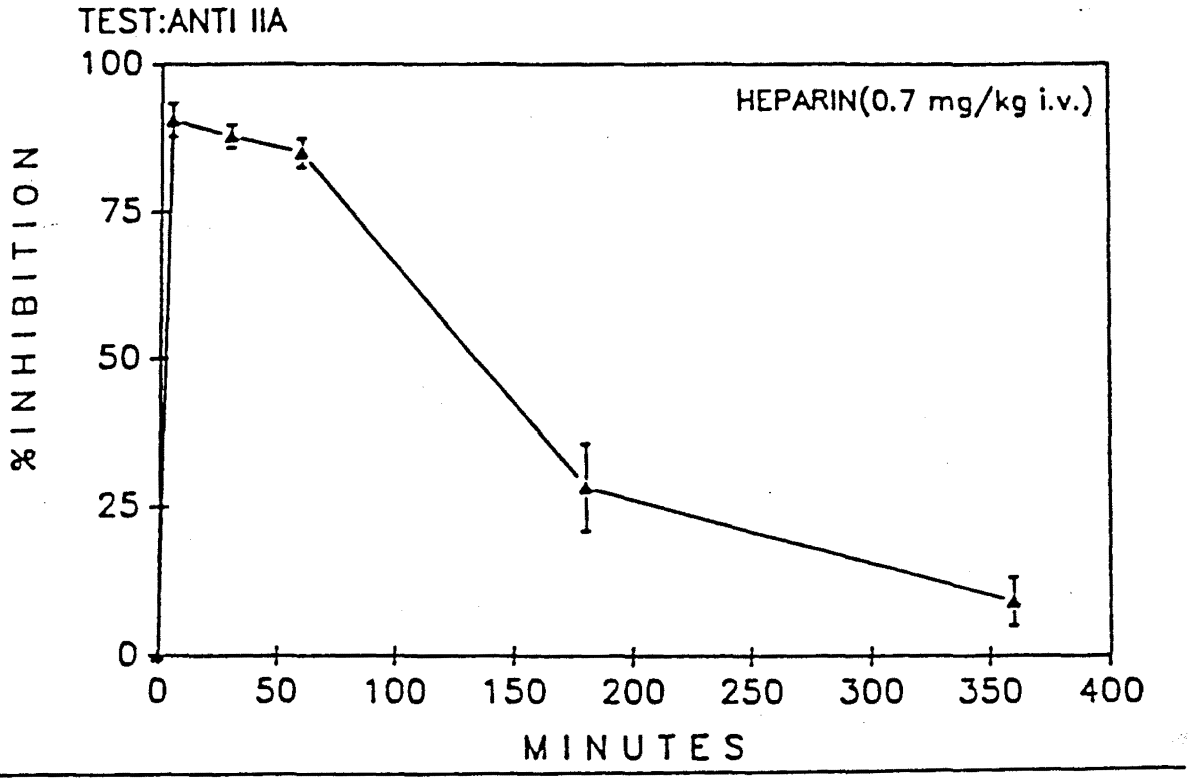


Figure 53. Four groups of primates were included in this study. The first group (N = 4) was administered heparin (0.7 mg/kg i.v.), (upper panel). The other three groups (N = 5) received heparin (0.7 mg/kg i.v.) followed by protamine sulfate (0.7, 1.4, or 2.1 mg/kg i.v.), five minutes after heparin administration (lower panel). Blood samples from each of these 4 groups were drawn at 0, 5, 30, 60, 180 and 360 minutes. An additional blood sample was obtained at 15 minutes from primates which received protamine at the 5 minute time period. Antiprotease activity was determined using an amidolytic anti IIa assay. Each data point represents a mean ± S.E.

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF HEPARIN IN PRIMATES



WITH PROTAMINE SULFATE

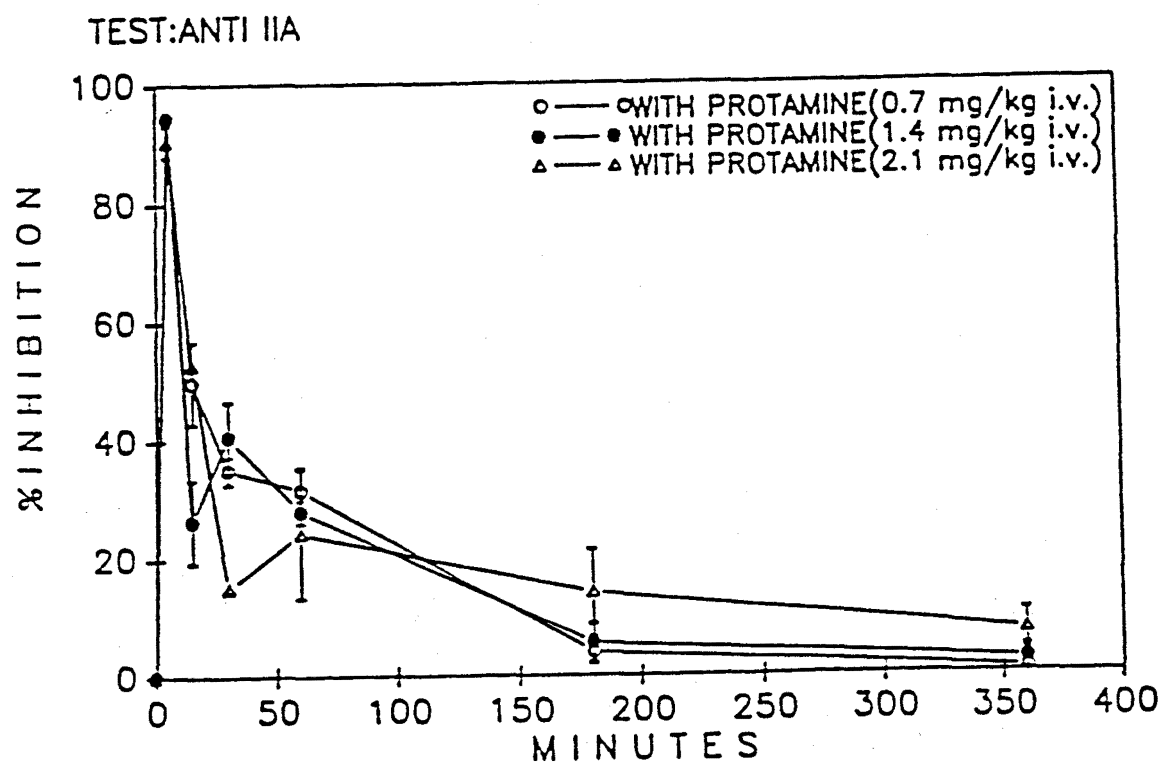
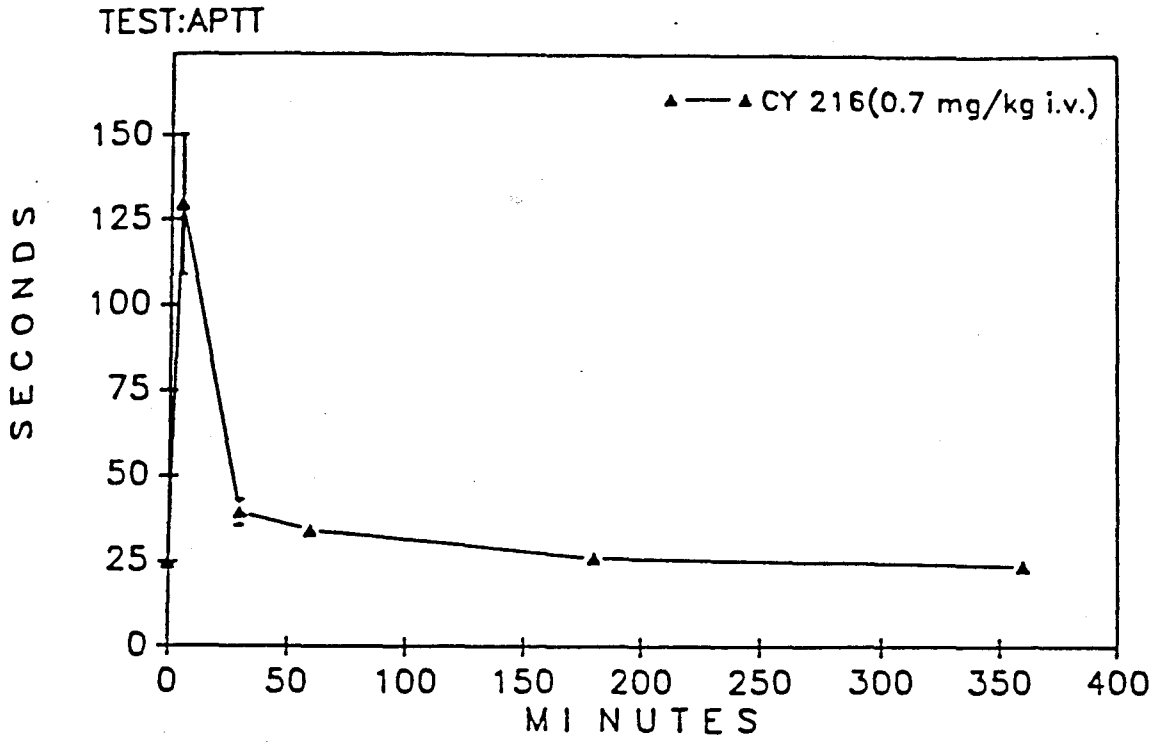


Figure 54. Four groups of primates were included in this study. The first group (N = 4) was administered CY 216 (0.7 mg/kg i.v.) (upper panel). The other three groups (N = 5) received CY 216 (0.7 mg/kg i.v.) followed by protamine sulfate (0.7, 1.4, or 2.1 mg/kg i.v.), five minutes after heparin administration (lower panel). Blood samples from each of these 4 groups were drawn at 0, 5, 30, 60, 180 and 360 minutes. An additional blood sample was obtained at 15 minutes from primates which were treated with protamine at the 5 minute time period. Anticoagulant activity activity was determined using the APTT. Each data point represents a mean ± S.E.

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY
OF CY 216 IN PRIMATES



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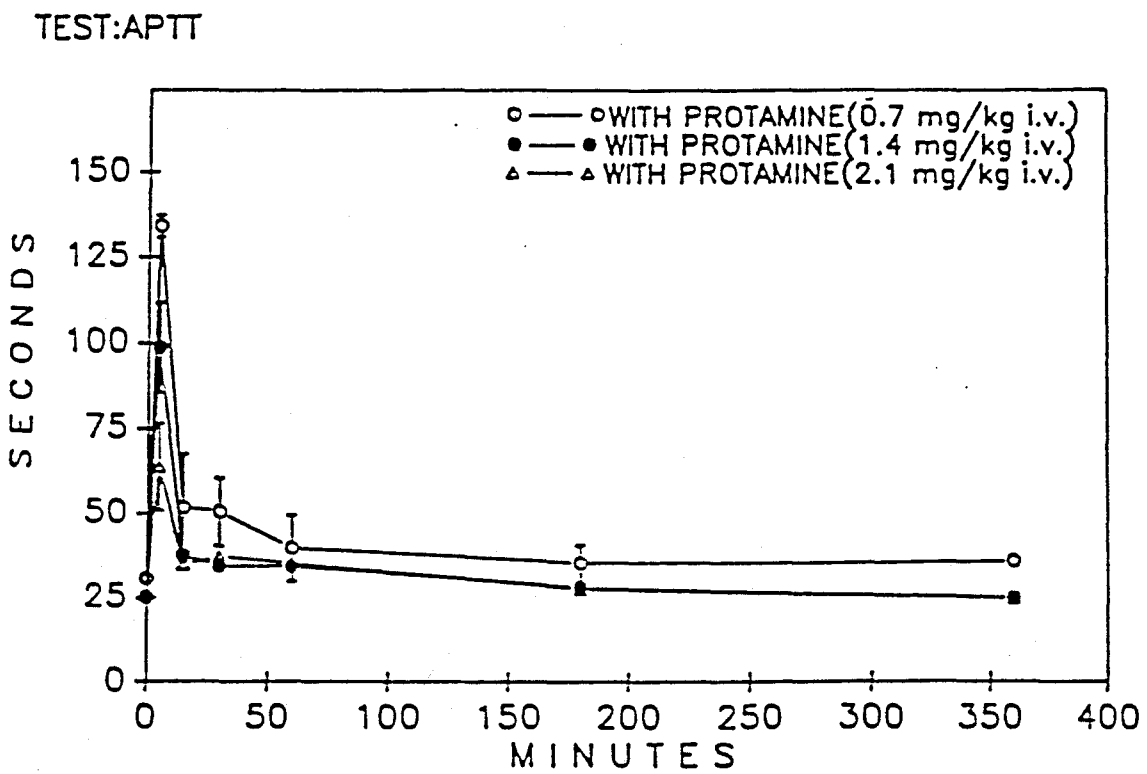
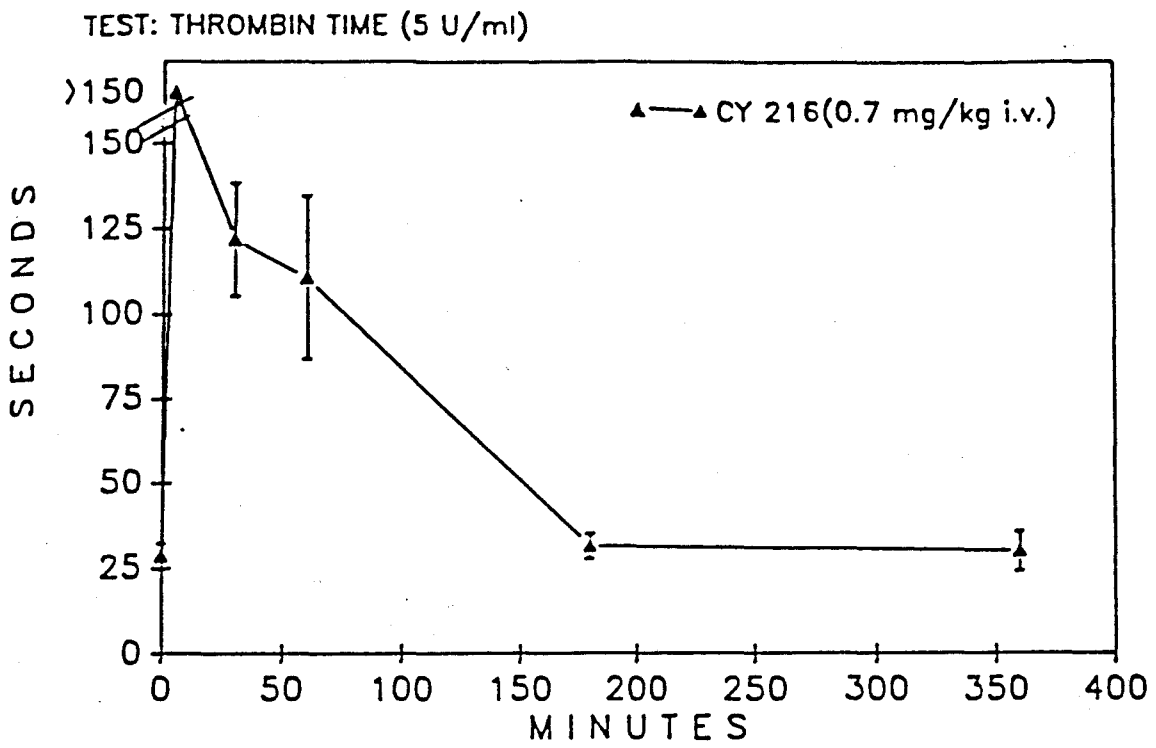


Figure 55. Four groups of primates were included in this study. The first group (N = 4) was administered CY 216 (0.7 mg/kg i.v.), (upper panel). The other three groups (N = 5) received CY 216 (0.7 mg/kg i.v.) followed by protamine sulfate (0.7, 1.4, or 2.1 mg/kg i.v.), five minutes after heparin administration (lower panel). Blood samples from each of these 4 groups were drawn at 0, 5, 30, 60, 180, and 360 minutes. An additional blood sample was obtained at 15 minutes from primates which were treated with protamine at the 5 minute time period. Anticoagulant activity was determined using the thrombin time. Each data point represents a mean ± S.E.

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF CY 216 IN PRIMATES



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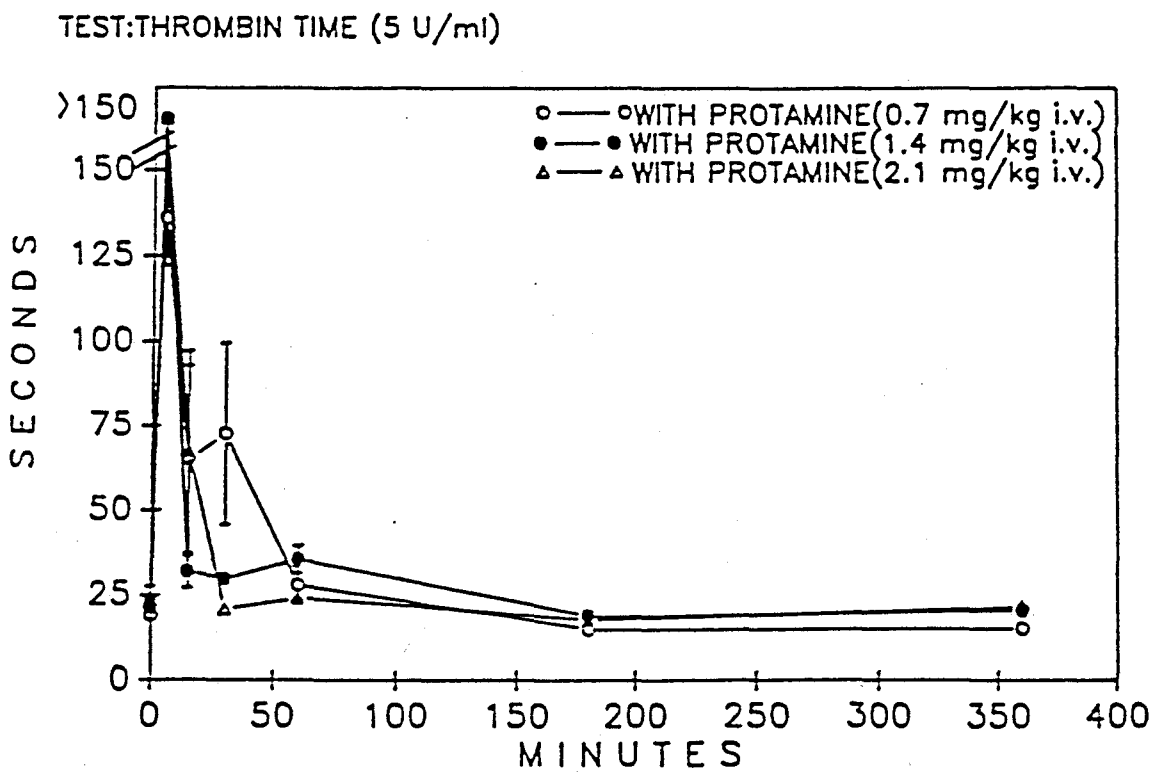
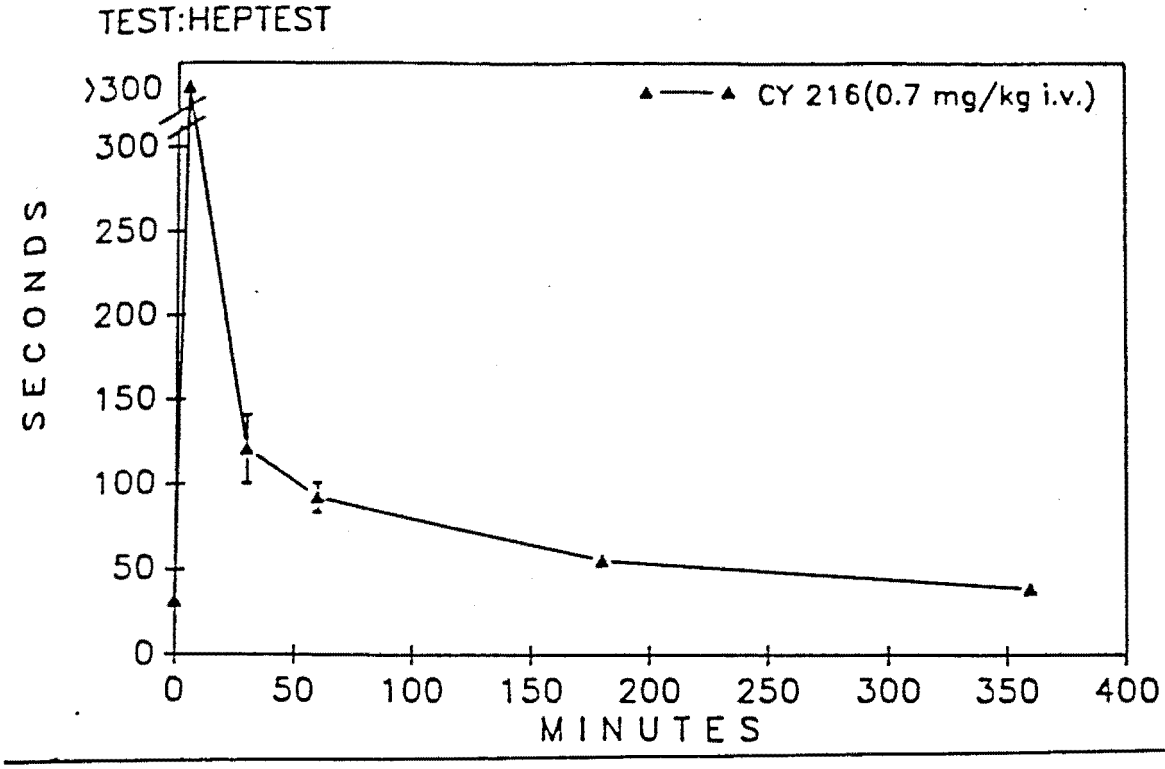


Figure 56. Four groups of primates were included in this study. The first group (N = 4) was administered CY 216 (0.7 mg/kg i.v.), (upper panel). The other three groups (N = 5) received CY 216 (0.7 mg/kg i.v.) followed by protamine sulfate (0.7, 1.4, or 2.1 mg/kg i.v.), five minutes after heparin administration (lower panel). Blood samples from each of these 4 groups were drawn at 0, 5, 30, 60, 180, and 360 minutes. An additional blood sample was obtained from primates which were treated with protamine at the 5 minute time period. Antiprotease activity was determined by the Heptest^{R}. Each data point represents a mean ± S.E.

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY
OF CY 216 IN PRIMATES



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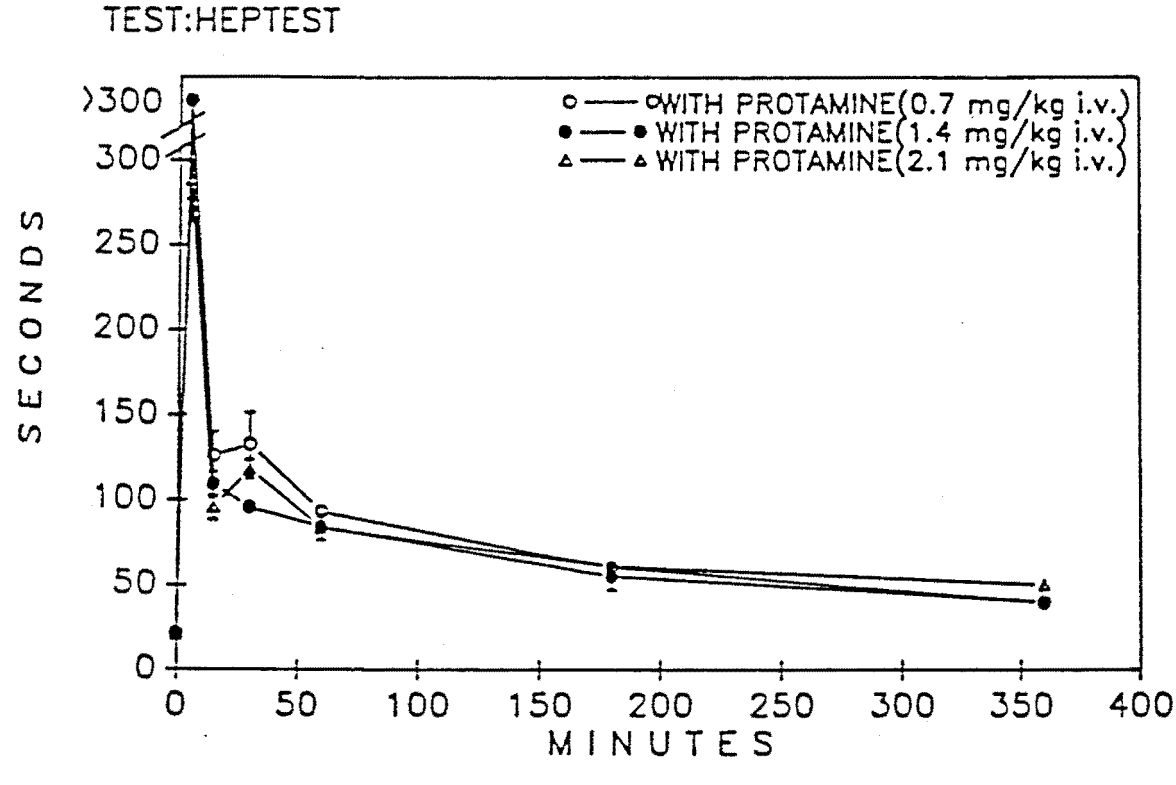


Figure 57. Four groups of primates were included in this study. The first group (N = 4) was administered CY 216 (0.7 mg/kg i.v.), (upper panel). The other three groups (N = 5) received CY 216 (0.7 mg/kg i.v.) followed by protamine sulfate (0.7, 1.4, or 2.1 mg/kg i.v.), five minutes after heparin administration (lower panel). Blood samples from each of these 4 groups were drawn at 0, 5, 30, 60, 180 and 360 minutes. An additional blood sample was obtained at 15 minutes from primates which were treated with protamine at the 5 minute time period. Antiprotease activity was determined using an amidolytic anti Xa assay. Each data point represents a mean ± S.E. Statistical anlaysis was performed by a two way ANOVA:

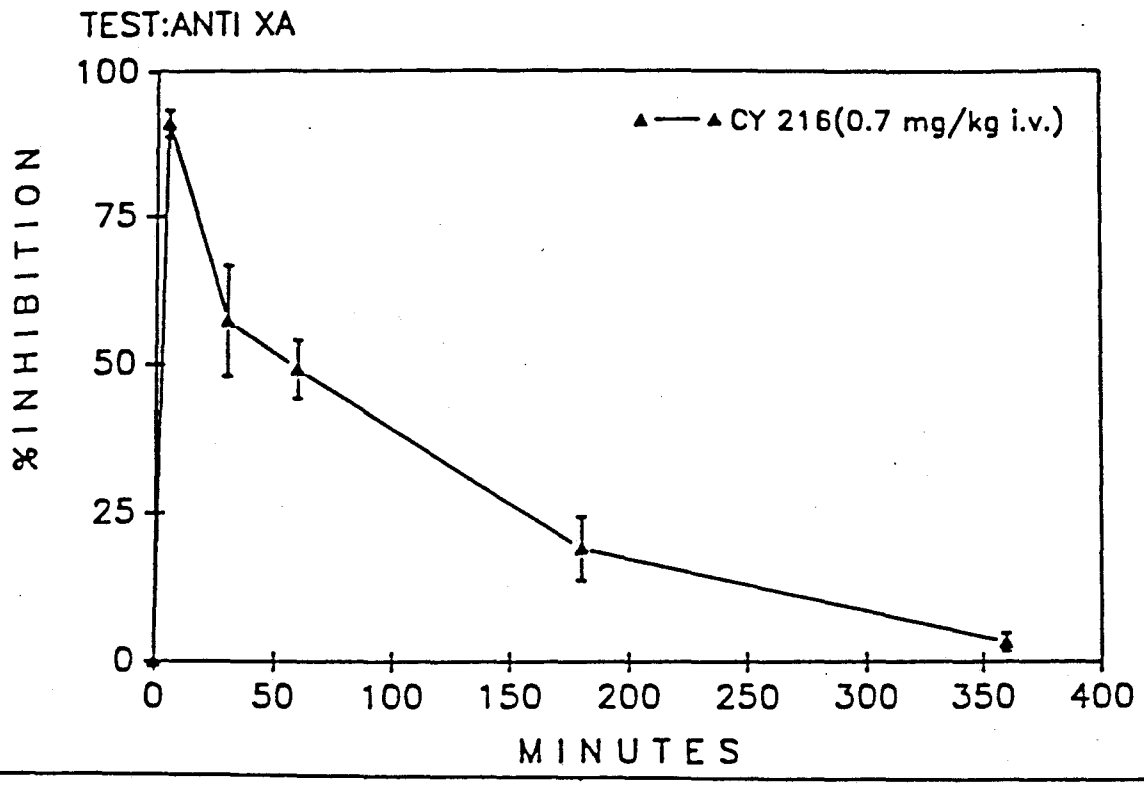
Factor A (time): $F(4,60) = 48.857$ $p < .001$

Factor B (protamine dose): $F(4,60) = 32.178$ $p < .001$

Factor A x B: $F(8,60) = .737$ $p > .50$ N.S.

followed by a Newman-Keuls multiple comparison test. * represents a significant difference from the CY 216 (0.7 mg/kg i.v.) and protamine (2.1 mg/kg i.v.) group.

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF CY 216 IN PRIMATES



WITH PROTAMINE SULFATE

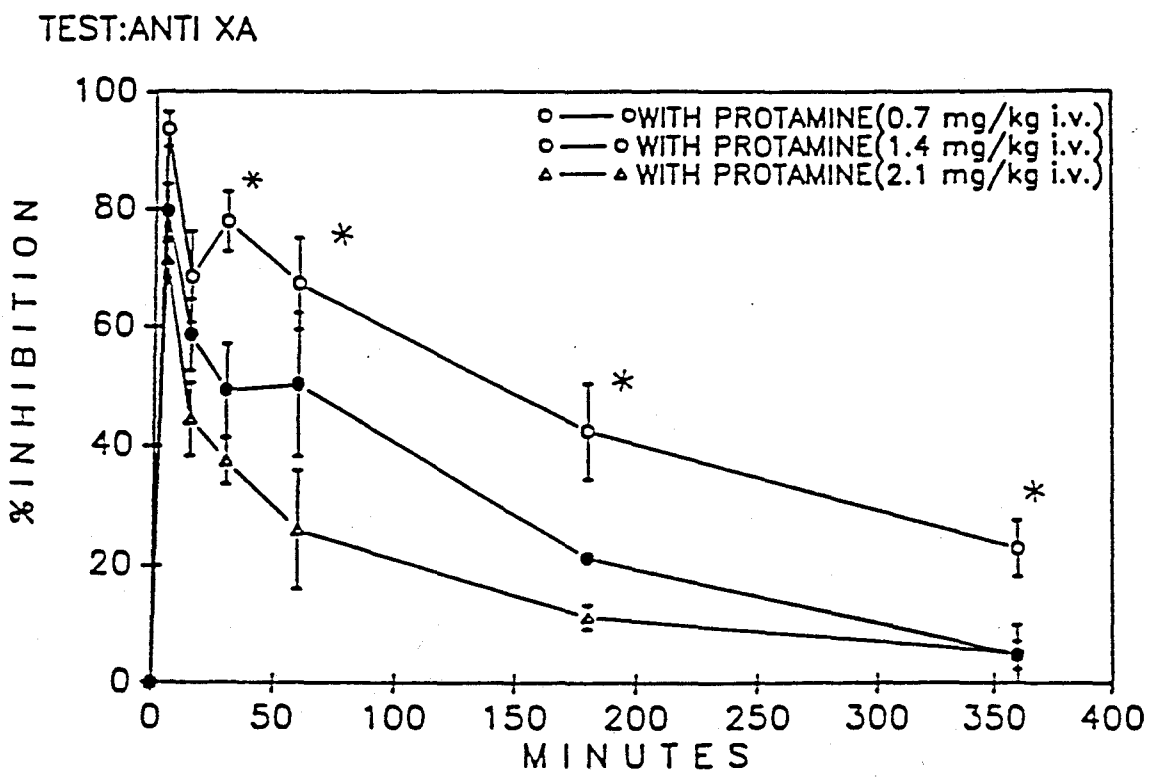


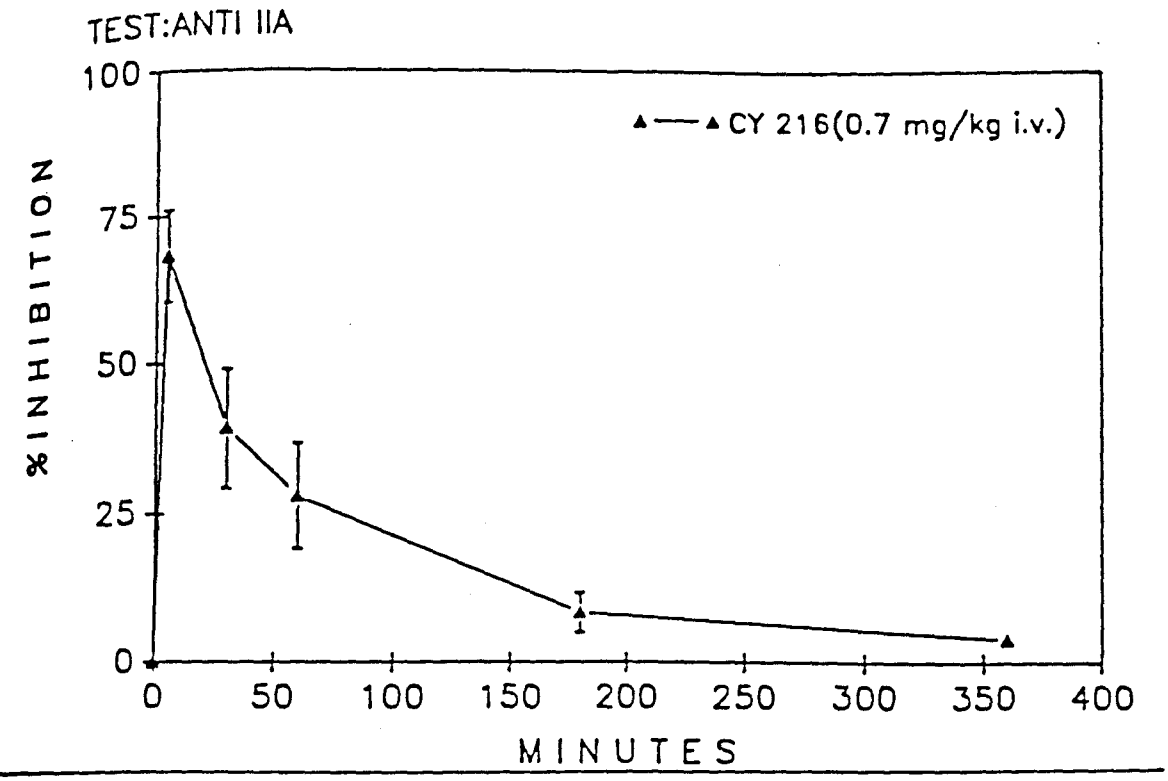
Figure 58. Four groups of primates were included in this study. The first group (N = 4) was administered CY 216 (0.7 mg/kg i.v.), (upper panel). The other three groups (N = 5) received CY 216 (0.7 mg/kg i.v.) followed by protamine sulfate (0.7, 1.4, or 2.1 mg/kg i.v.), five minutes after heparin administration (lower panel). Blood samples from each of these 4 groups were drawn at 0, 5, 30, 60, 180 and 360 minutes. An additional blood sample was obtained at 15 minutes from primates which were treated with protamine at the 5 minute time period. Antiprotease activity activity was determined using an amidolytic anti IIa assay. Each data point represents a mean ± S.E. Statistical analysis was performed by a two way ANOVA:

Factor A (time): $F:(3,48) = 1056.337$ $p<.001$

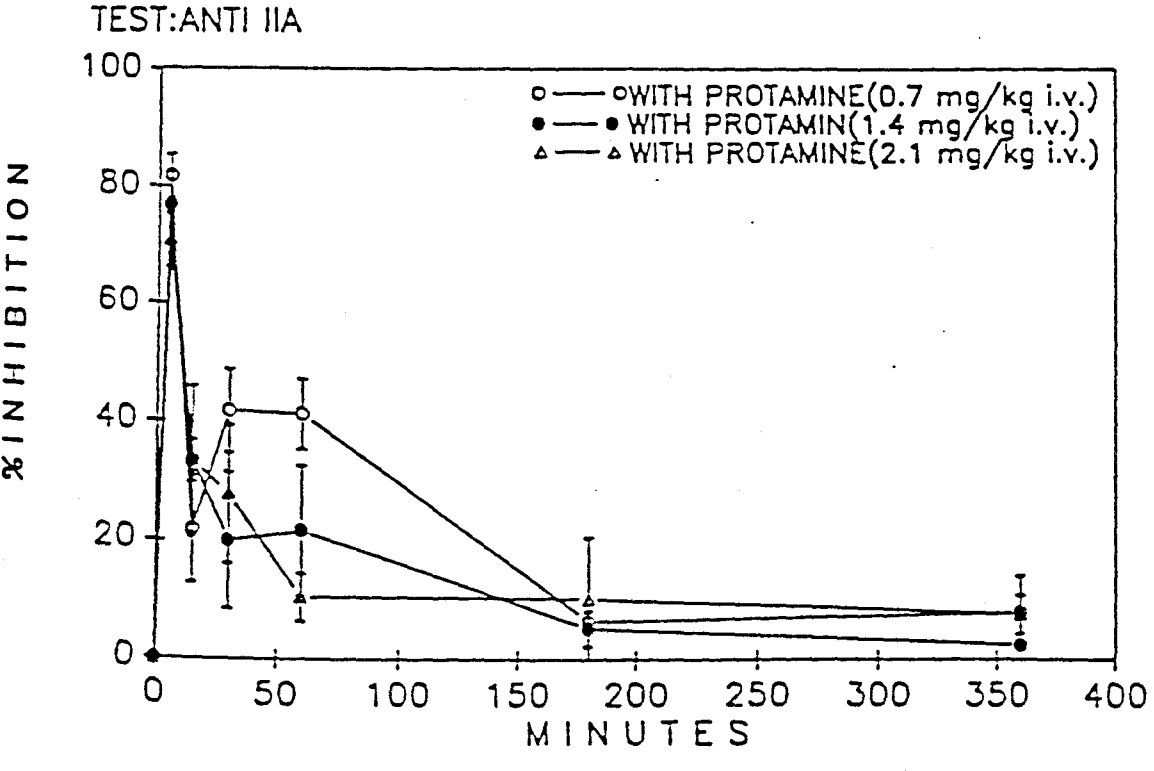
Factor B (protamine dose): $F(2,48) = .033$ $p>.50$ N.S.

Factor A x B: $F(6,48) = .267$ $p>.50$ N.S.

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF CY 216 IN PRIMATES



WITH PROTAMINE SULFATE



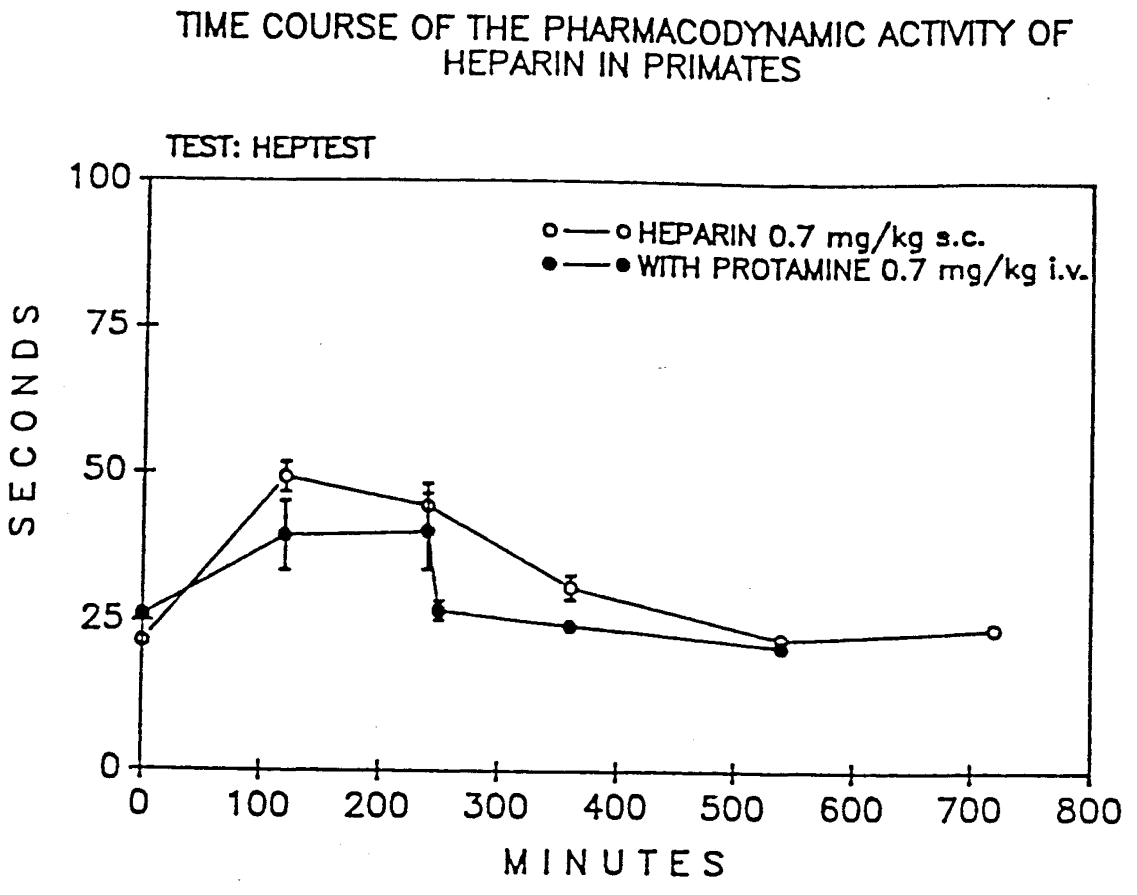


Figure 59. Two groups of primates were included in this study. The first group (N = 5), (open circles) was administered heparin (0.7 mg/kg s.c.). The second group (N = 4), (closed circles) received heparin (0.7 mg/kg s.c.) followed by protamine sulfate (0.7 mg/kg i.v.) 240 minutes after heparin administration. Blood samples were drawn at 0, 120, 240, 360 and 540 minutes. An additional blood sample was obtained at 245 minutes from primates which were treated with protamine. A final blood sample was drawn at 720 minutes from the heparin group. Antiprotease activity was determined using the Heptest^{R}. Each data point represents a mean ± S.E.

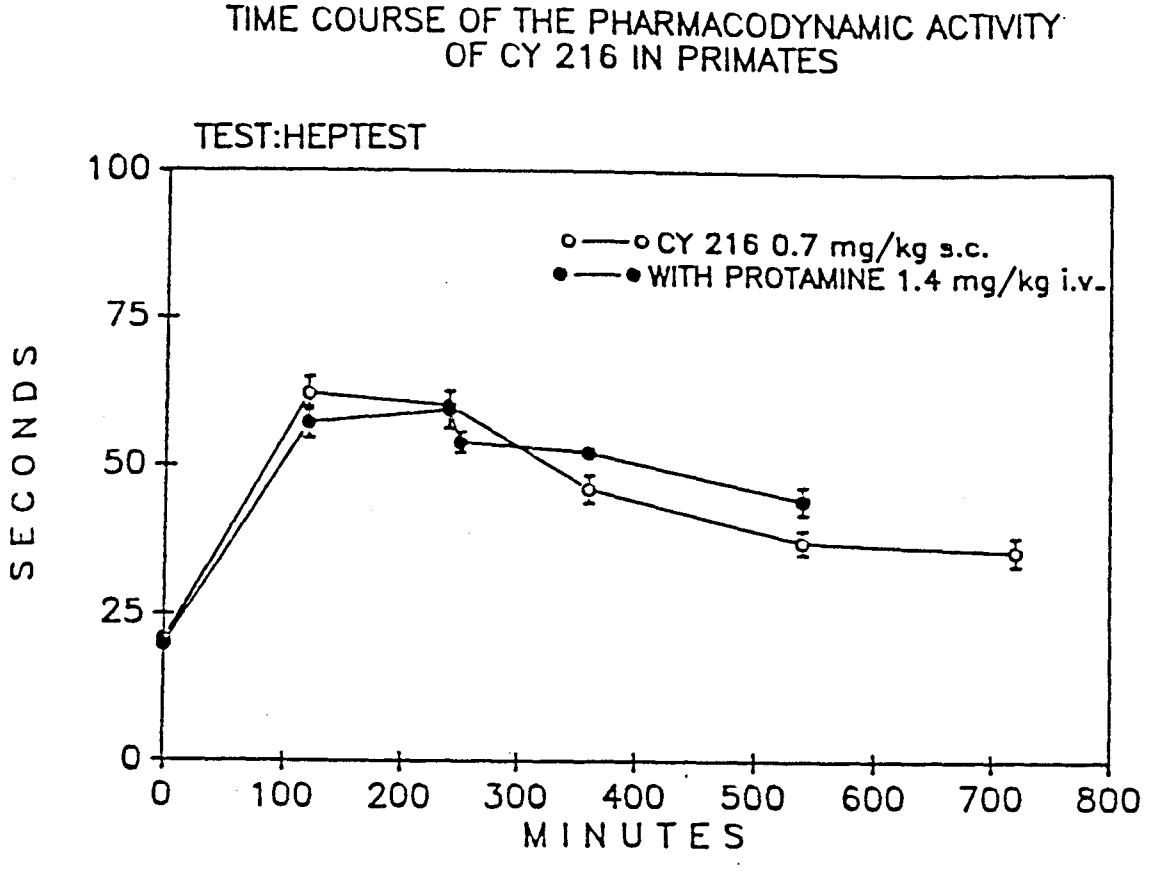


Figure 60. Two groups of primates were included in this study. The first group (N = 5), (open circles) was administered CY 216 (0.7 mg/kg s.c.). The second group (N = 4), (closed circles) received CY 216 (0.7 mg/kg s.c.) followed by protamine sulfate (1.4 mg/kg i.v.) 240 minutes after heparin administration. Blood samples were drawn at 0, 120, 240, 360 & 540 minutes. An additional blood sample was obtained at 245 minutes from primates which were treated with protamine. A final blood sample was drawn at 720 minutes from the heparin group. Antiprotease activity was determined using a clot based assay (Heptest^{R}). Each data point represents a mean ± S.E.

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF HEPARIN IN PRIMATES

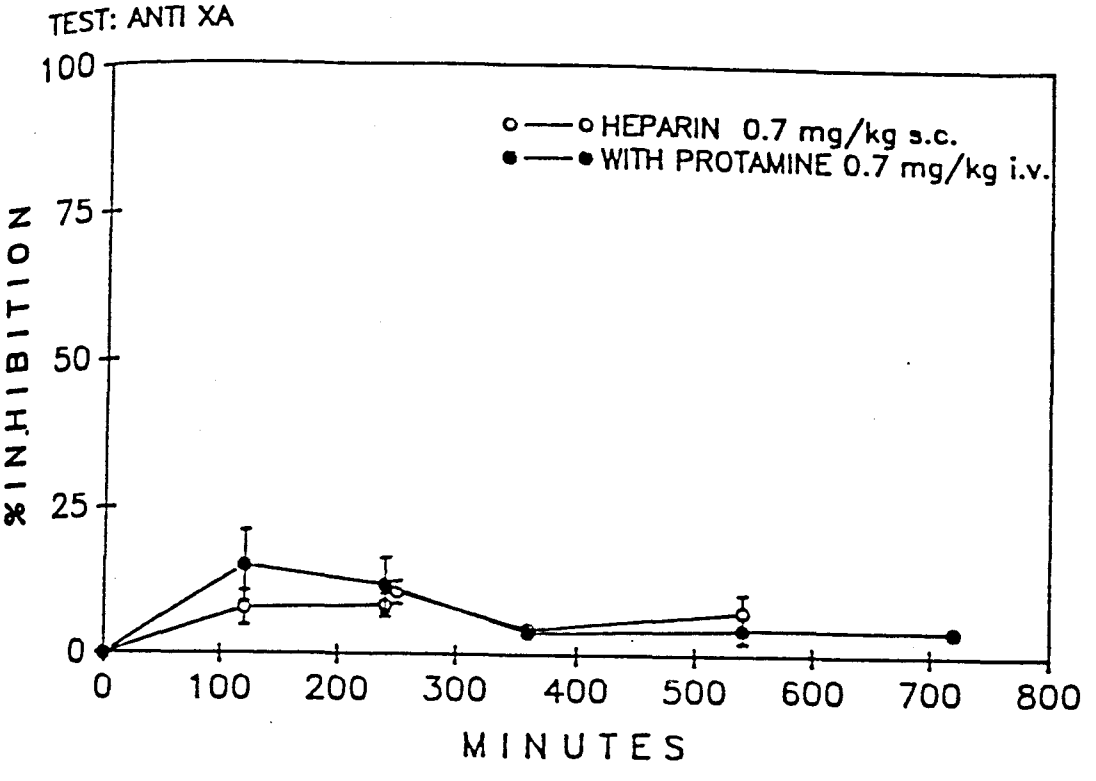


Figure 61. Two groups of primates were included in this study. The first group (N = 5), (open circles) was administered heparin (0.7 mg/kg s.c.). The second group (N = 4), (closed circles) received heparin (0.7 mg/kg s.c.) followed by protamine sulfate (0.7 mg/kg i.v.) 245 minutes after heparin administration. Blood samples were drawn at 0, 120, 240, 360 and 540 minutes. An additional blood sample was obtained at 245 minutes from primates which received protamine. A final blood sample was drawn at 720 minutes from the heparin group. Antiprotease activity was determined using an amidolytic anti Xa assay. Each data point represents a mean ± S.E.

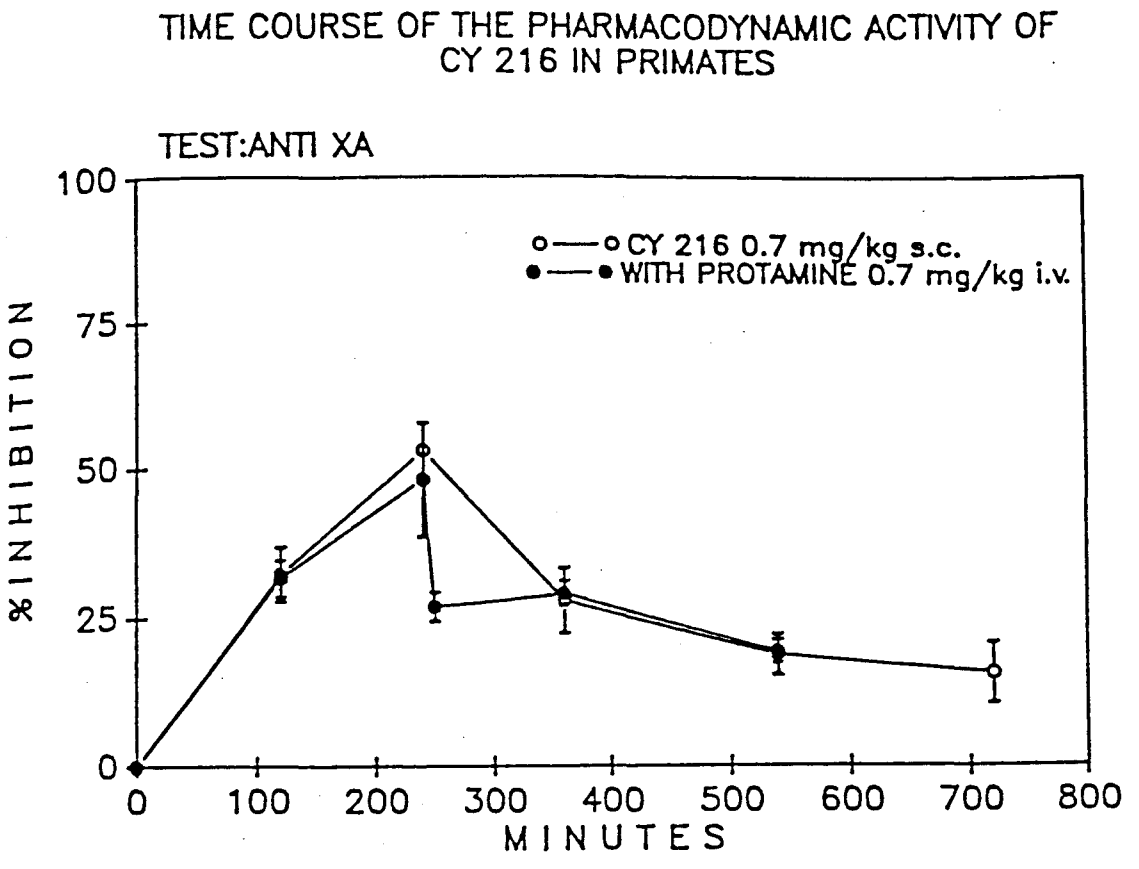


Figure 62. Two groups of primates were included in this study. The first group (N = 5), (open circles) was administered CY 216 (0.7 mg/kg s.c.). The second group (N = 4), (closed circles) received CY 216 (0.7 mg/kg s.c.) followed by protamine sulfate (0.7 mg/kg i.v.) 240 minutes after heparin administration. Blood samples were drawn at 0, 120, 240, 360 and 540 minutes. An additional blood sample was obtained at 240 minutes for primates which received protamine. A final blood sample was drawn at 720 minutes from the heparin group. Antiprotease activity was determined using an amidolytic anti Xa assay. Each data point represents a mean ± S.E.

CHAPTER IX

BIBLIOGRAPHY

Ablidgaard, U.: Inhibition of the Thrombin-Fibrinogen Reaction by Antithrombin 3 Studied by N-Terminal Analysis. Scandinavian Journal of Clinical Laboratory Investigation, 20:205-16, 1967.

Al-Mondhiry, H., Pierce, W.S., and Basarab, R.M.: Protamine-Induced Thrombocytopenia and Leukopenia. Thrombosis and Haemostasis, 53:(1) 60-64, 1985.

Andersson, L.O., Barrowcliffe, T.W., Holmer, E., Johnson, E.A. and Sims, G.E.C.: Anticoagulant Properties of Heparin Fractionated by Affinity Chromatography on Matrix Bound Antithrombin III and by Gel Filtration. Thrombosis Research, 9:575, 1976.

Andersson, L.O., Barrowcliffe, T.W., Holmer, E., Johnson, E.A. and Soderstrom, G.: Molecular Weight Dependency of the Heparin Potentiated Inhibition of Thrombin and Activated Factor X. Effect of Heparin Neutralization in Plasma. Thrombosis Research, 15:531-541, 1979.

Ando, T., Yamasaki, M., and Suzuki, K.: Protamine - Isolation, Characterization, Structure and Function, Springer, Berlin, Heidelberg, New York, 1973.

Andrew, M., Ofosu, F., Fernandez, F., Jefferies, A., Hirsh, J., Mitchell, L. and Buchanan, M.R.: A Low Molecular Weight Heparin Alters the Fetal Coagulation System in the Pregnant Sheep. Thrombosis and Haemostasis, 55:342-346, 1986.

Arneson, H., Engebretsen, L.F., Ugland, O.M., Seljeflot, I. and Kierulf, P.: Increased Fibrinolytic Activity After Surgery Induced By Low Dose Heparin. Thrombosis Research, 45:553-559, 1987.

Arnott, S. and Winter, W.T.: Details of Glycosaminoglycan Conformations and Intermolecular Interactions. Federation Proceedings, 36:(1) 73-77, 1977.

Atha, D.H., Lormeau, J.C., Petitou, M., Rosenberg, R.D., and Choay, J.: Contribution of 3-O- and 6-O-Sulfated Glucosamine Residues in the Heparin-Induced Conformational Change in Antithrombin III. Biochemistry, 26:(20) 6454-61, 1987.

Barlow, G.H. and Petracek, F.J.: A Physical Comparison of Low Molecular Weight Heparins Prepared by Different Methods. Research Communications in Chemical Pathology and Pharmacology, 53:(2) 241-244, 1986.

Barrowcliffe, T.W., Merton, R.E., Havercroft, S.J., Thunberg, L., Lindahl, U., and Thomas, D.P.: Low-Affinity Heparin Potentiates the Action of High Affinity Heparin Oligosaccharides. Thrombosis Research, 34:125-133, 1984.

Barzu, T., Molho, P., Tobelem, G., Petitou, M. and Caen, J.: Binding and Endocytosis of Heparin by Human Endothelial Cells in Culture. Biochemica et Biophysica Acta, 845:196-203, 1985.

Barzu, T., van Rijn, J.L.M.L., Petitou, M., Tobelem, G. and Caen, J.P.: Heparin Degradation in the Endothelial Cell. Thrombosis Research, 47:601-609, 1987.

Benayahu, D. and Aronson, M.: Comparative Study of Protamine Chloride and Sulfate in Relation to the Heparin Rebound Phenomenon. Thrombosis Research, 32:109-114, 1983.

Berqvist, D., Hedner, U., Sjorin, E. and Holmer E.: Anticoagulant Effects of Two Types of Low Molecular Weight Heparins Administered Subcutaneously. Thrombosis Research, 32:381-391, 1983.

Berqvist, D., Burmark, U.S., Frisell, J., Hallbrook, T., Lindblad, B., Risberg, B., Turngren, S. and Wallin, G.: Low Molecular Weight Heparin once Daily Compared with Conventional Low Dose Heparin Twice Daily. A Prospective Double-Blind Multicentre Trial on Prevention of Postoperative Thrombosis. British Journal of Surgery, 73, 204-298, 1986.

Bertoli, D. and Borelli, G. Peri- and Postnatal, Teratology and Reproductive Studies of a Low Molecular Weight Heparin in Rats. Arzneim-Forsch/Drug Research, 36:(II) 1260-1263, 1986.

Best, N., Sinosich, M.J., Teisner, B., Grudzinskas, J.G. and Fisher, M.: Complement Activation During Cardiopulmonary Bypass by Heparin-Protamine Interactions. British Journal of Anaesthesiology, 56:339-343, 1984.

Bianchini, P., Osima, B., Parma, B., Nader, H.B. and Dietrich, C.P.: Lack of Correlation Between "In Vitro" and "In Vivo" Antithrombotic Activity of Heparin Fractions and Related Compounds. Heparan Sulfate as an Antithrombotic Agent "In Vivo". Thrombosis Research, 40:597-607, 1985.

Bjoraker, D.G. and Ketcham, T.R.: Hemodynamic and Platelet Response to the Bolus Intravenous Administration of Porcine Heparin. Thrombosis and Haemostasis, 49:(1) 1-4, 1983.

Bjornsson, T.D., Wolfram, K.M. and Kitchell, B.B.: Heparin Kinetics Determined by Three Assay Methods. Clinical Pharmacology and Therapeutics, 32:104- 113, 1981.

Boneu, B., Buchanan, M.R., Cade, J.F., Van Ryn, J., Fernandez, F.F., Ofosu, F. and Hirsh, J.: Effects of Heparin, Its Low Molecular Weight Fractions and Other Glycosaminoglycans of Thrombus Growth In Vivo. Thrombosis Research, 40:81-89, 1985.

Boneu, B., Caranobe, C., Gabaig, A., Dupouy, D. and Sie, P.: Evidence for

a Saturable Mechanism of Disappearance of Standard Heparin in Rabbits. Thrombosis Research, 46:835-844, 1987.

Borelli, G. and Bertoli, D.: Toxic and Mutagenic Effects of a Low Molecular Weight Heparin in Rats. Arzneim-Forsch/Drug Research, 36(II):1256-1260, 1986.

Bourne, D.W.A., Triggs, E.J. and Eadie, M.J.: Pharmacokinetics for the Non-mathematical, MTP Press Limited, Lancaster, Australia, 1988.

Brace, L.D. and Fareed, J.: Heparin-Induced Platelet Aggregation: Dose/Response Relationships For A Low Molecular Weight Heparin Derivative (PK 10169) and Its Subfractions. Thrombosis Research, 42: 769-782, 1986.

Breddin, H.K., Wiedemann, R., Weichert, W: Entstehung von Thrombozytethromben In Vivo. Film N. C 1323 IWF, Gottingen, Publikation von W. Weichert, Publ. Wiss. Film, Sekt. Med., Ser. 5, 18/3, 1323, 1982.

Brufani, M., Corbelli, G., Mascellani, G. and Stanzani, L.: Stability of Heparin and Other Fractions of Glycosaminoglycan Sulfates in Human Digestive Juices. Journal of Pharmaceutical Sciences, 71:(1) 128-129, 1982.

Buchanan, M.R., Boneu, B., Ofosu, F. and Hirsh, J.: The Relative Importance of Thrombin Inhibition and Factor Xa Inhibition to the Antithrombotic Effects of Heparin. Blood, 65:(1) 198-201, 1985.

Bull, B.S., Korpman, R.A., Huse, W.M., and Briggs, B.D.: Heparin Therapy During Extracorporeal Circulation I. Problems Inherent in Existing Heparin Protocols. The Journal of Thoracic and Cardiovascular Surgery, 69:(5) 672-684, 1975.

Bull, B.S., Huse, W.H., Brauer, F.S. and Korpman, R.A.: Heparin Therapy During Extracorporeal Circulation II. The Use of a Dose Response Curve to Individualize Heparin and Protamine Dosage. Journal of Thoracic and Cardiovascular Surgery, 69:(6), 685-689, 1975.

Cade, J.F., Buchanan, M.R., Boneu, B. and Ockelford, P.: A Comparison of the Antithrombotic and Haemorrhagic Effects of Low Molecular Weight Heparin Fractions: The Influence of the Method of Preparation. Thrombosis Research, 35:613-625, 1984.

Cadroy, Y., Dol, F., Caranobe, C., Petitou, M., Lormeau, J.C., Sie, P., Choay, J. and Boneu B. Standard Heparin Enhances the Antithrombotic Activity of Dermatan Sulfate in the Rabbit But CY 216 Does Not. Thrombosis and Haemostasis, 59:(2) 295-8, 1988.

Campbell, F.W., Goldstein, M.F. and Atkins, P.C.: Management of the Patient with Protamine Hypersensitivity for Cardiac Surgery. Anesthesiology, 61:761-764, 1984.

Carter, C.J., Kelton, J.G., Hirsh, J., Cerskus, A., Santos, A.V. and Gent,

M. The Relationship Between the Hemorraghic and Antithrombotic Properties of Two Low Molecular Weight Heparins in Rabbits. Blood, 59:1239-1245, 1982.

Castenda, A.R.: Must Heparin Be Neutralized Following Open-Heart Operations? Journal of Thoracic and Cardiovascular Surgery, 52:716, 1966.

Casu, B., Oreste, P., Torri, G., Zoppetti, G., Choay, J., Lormeau, J.C., Petitou, M. and Sinay, P.: The Structure of Heparin Oligosaccharide Fragments with High Anti (Factor Xa) Activity Containing the Minimal Antithrombin III-Binding Sequence. Biochemical Journal, 197:599-609, 1981.

Casu, B.: Structure of Heparins and Their Fragments. Nouveau Revue Hematologie, 26:211-219, 1984.

Casu, B.: Structure and Biological Activity of Heparin. Advances in Carbohydrate, Chemistry and Biochemistry, 43, 51-134, 1985.

Caulfield, J.P., Lewis, R.A., Hein, A., and Austen, K.F.: Secretion in Dissociated Human Pulmonary Mast Cells. Evidence for Solubilization of Granule Contents Before Discharge. Journal of Cell Biology, 85:299-312, 1980.

Cella, G., Myers, C.J., Prosdocimi, M. and Sasahara, A.A.: Human Platelet Factor 4 and Protamine Sulfate Interaction with Glycosaminoglycans in the Rabbit. European Journal of Clinical Investigation, 17:548-554, 1987.

Chandler, W.F., Ercius, M.S., Ford, J.W., LaBond, V. and Burkel, W.E.: The Effect of Heparin Reversal After Carotid Endarterectomy in the Dog. Journal of Neurosurgery, 56:97-102, 1982.

Chargaff, E. and Olson, K.B.: Studies on the Chemistry of Blood Coagulation VI. Studies on the Action of Heparin and Other Anticoagulants. The Influence of Protamine on the Anticoagulant Effect In Vivo. Journal of Biological Chemistry, 122:153 1937.

Charles, A.F. and Scott, D.A.: Studies on Heparin III. The Purification of Heparin. Journal of Biological Chemistry, 102:425 1933.

Chernoff, A.: Anaphylactic Reactions Following Injection of Heparin. New England Journal of Medicine, 242:315, 1950.

Choay, J., Lormeau J.C., Petitou M., Sinay P., Casu B., Oreste P., Torri G. and Gatti G.: Anti Xa Active Heparin Oligosaccharides. Thrombosis Research, 18:3-4, 1980.

Choay, J., Petitou, M., Lormeau, J.C., Jacquinet, J.C. and Sinay, P. Heparin Antithrombin III Binding Sequence: Approach of Structure/Activity Relationship by Chemical Synthesis (Abstr) Thrombosis and Haemostasis, 50:360 1983.

Choay, J., Petitou, M., Lormeau, J.C., Sinay, P., Casu, B. and Gatti G.: Structure-Activity Relationship in Heparin: A Synthetic Pentasaccharide With High Affinity For Antithrombin III and Eliciting High Anti-Factor Xa Activity. Biochemical and Biophysical Research Communications, 116:(2)

492-499, 1983.

Choay, J. and Petitou, M.: The Chemistry of Heparin: A Way to Understand Its Mode of Action. The Medical Journal of Australia, 144:Hs 7-10, 1986.

Cifonelli, J.A.: Nitrous Acid Depolymerization of Glycosaminoglycans. Methods in Carbohydrate Chemistry, 7:139-141, 1976.

Cobel-Geard, R.J. and Hassouna, H.I.: Interaction of Protamine Sulfate with Thrombin. American Journal of Hematology, 14:227-233, 1983.

Cocchetto, D. and Bjornsson, T.D.: Progress Toward an Understanding of the Anticoagulant Effect and Pharmacokinetics of Heparin. Pharmacy International, 5:(1) 7-11, 1984.

Cohen, J.A., Frederickson, E.L. and Kaplan, J.A.: Plasma Heparin Activity and Antagonism During Cardiopulmonary Bypass and Hypothermia. Anesthesia and Analgesia, 56:(4) 564-568, 1977.

Colman, R.W., Hirsh, J., Marder, V.J. and Salzman, E.W.: Hemostasis and Thrombosis:Basic Principles and Clinical Practice. J.B. Lippincott Company, Philadelphia, 1987.

Conley, C.L., Morse, W.I. and Stewart, E.E.: Thromboplastic Factors in Estimation of Prothrombin Concentration. American Journal of the Medical Sciences, 215:158-169, 1948.

Conrad, H.E.: The Acid Lability of the Glycosidic Bonds of L-Iduronic Acid Residues in Glycosaminoglycans. Biochemical Journal, 191:355-363, 1981.

Conzen, P.F., Habazetti, H., Gutmann, R., Hobbhahn, J., Goetz, A.E., Peter, K., and Brendel, W.: Thromboxane Mediation of Pulmonary Hemodynamic Responses After Neutralization by Protamine in Pigs. Anesthesia and Analgesia, 68:25-31, 1989.

Copley, A.L.: Editorial:Bleeding time. Progress in Hemostasis and Thrombosis, 4:1-23, 1974.

Coulon, C., Freysz, M., Jabouef, R., David, M.: Appreciation of Optimal Loading At the End of Cardiopulmonary Bypass: "The Protamine Sulfate Test". International Journal of Artificial Organs, 5:387, 1982.

Crafoord, C.: Heparin and Post-Operative Thrombosis. Acta Chirugica Scand, 82:319, 1939.

Dahan, D., Houlbert, D., Caulin, C., Cuzin, C., Viltart B., Woler, M., and Segresta, A.: Prevention of Deep Vein Thrombosis in Elderly Medical Patients By a Low Molecular Weight Heparin: A Randomized Double-Blind Trial. Haemostasis, 16:159-164, 1986.

Danishefsky, I., Eiber, H.B.: Studies on the Metabolism of Heparin.

Archives of Biochemistry, 85:53, 1959.

Dawes, J. and Pepper, D.S.: Catabolism of Low Dose Heparin in Man. Thrombosis Research, 14:(6) 845-60, 1979.

Dawes, J. and Pepper, D.S.: A Sensitive Competitive Binding Assay for Exogenous and Endogenous Heparins. Thrombosis Research, 27:387-396, 1982.

Dawes, J. and Pumphrey, C.W., Mclaren, K.M., Prowse, C.V. and Pepper, D.S.: The In Vivo Release of Human Platelet Factor 4 by Heparin. Thrombosis Research, 27:65-76, 1982.

Dawes, J., Bara, L., Billaud, E. and Samama, M.: Relationship Between Biological Activity and Concentration of a Low Molecular Weight Heparin (PK 10169) and Unfractionated Heparin after Intravenous and Subcutaneous Administration. Haemostasis, 16:116-122, 1986.

Dawes, J. Absorption of Heparin, LMW Heparin and SP54 After Subcutaneous Injection Assessed By A Competitive Binding Assay. Thrombosis Research, 44:683-693, 1986.

de Swart, C.A.M., Nijmeyer, B., Roelofs, J.M.M and Sixma, J.J. Kinetics of Intravenously Administered Heparin in Normal Humans. Blood, 60:(6) 1251-1258, 1982.

de Swart, C.A.M., Nijmeyer, B., Andersson, L.O., Holmer, E., Sixma, J.J. and Bouma, B.N.: Elimination of Intravenously Administered Radiolabelled Antithrombin III and Heparin in Humans. Thrombosis Haemostasis, 52:(1) 66-70, 1984.

de Prost, D.: Heparin Fractions and Analogues: A New Therapeutic Possibility for Thrombosis. Trends in Pharmaceutical Sciences, 3:496-500, 1986.

Dejana, E., Quintana, A., Callioni, A. and DeGaetano, G.: Bleeding Time In Laboratory Animals. II- A Comparison of Different Assay Conditions In Rats. Thrombosis Research, 15:191-197, 1979.

Dejana, E.: Quintana A., Callioni A. and DeGaetano G. Bleeding Time In Laboratory Animals. III- Do Tail Bleeding Times in Rats Only Measure a Platelet Defect? (The Aspirin Puzzle). Thrombosis Research, 15:199-207, 1979.

Dejana, E., Villa, S. and De Gaetano, G.: Bleeding Time in Rats:A comparison of Different Experimental Conditions. Thrombosis and Haemostasis, 48(1), 108-111, 1982.

Denton, J., Lane, D.A., Slater, A., Thronberg, L. and Lindahl, U.: Size of Heparin Oligosaccharides Required for Antithrombin Activity and Interaction with Platelet Factor 4 (PF-4). Thrombosis and Haemostasis, 50:692, 1983.

Di Stefano, J., Landaw, E.M.: Multiexponential, Multicompartmental, and

Noncompartmental Modeling. I. Methodological Limitations and Physiological Interpretations. Modeling Methodology Forum, The American Physiological Society, 1984.

Dietrich, C.P., Nader, N.B. and McDuffie, N.M.: Electrofocussing of Heparin: Presence of 21 Monomeric and Dimeric Molecular Species in Heparin Preparations. Annals of the Brazilian Academy of Science, 47:301-309, 1975.

Diness, V. and Ostergaard, P.B.: Neutralization of a Low Molecular Weight Heparin (LHN-1) and Conventional Heparin by Protamine Sulfate in Rats. Thrombosis and Haemostasis, 56:(3) 318-322, 1986.

Doutremepuich, C., Bonini, F., Toulemonde, F., Bertrand, H., Bayrou, B. and Quilichini, R.: In Vivo Neutralization of Low Molecular Weight Heparin Fraction CY 216 by Protamine. Seminars in Thrombosis and Hemostasis, 11:(3) 318-322, 1985.

Doutremepuich, C., Toulemonde, F., Bousquet, F. and Bonini, F.: Comparison of the Haemorrhagic Effects of Unfractionated Heparin and a Low Molecular Weight Heparin Fraction (CY 216) in Rabbits. Thrombosis Research, 43:691-695, 1986.

Edwards, H.E., Navaratman, S., Allen, J.C. and Phillips, G.O.: The Determination of the Heparin Neutralising Capacity of Protamine Using Acridine Orange Fluorescence. Thrombosis and Hemostasis, 43:(2), 108-111, 1980.

Eika, C. and Godal, H.C.: Thrombocytopenia in Rabbits Induced by Heparin, Protamine and Polybrene. Scandinavian Journal of Haematology, 8:481-486, 1971.

Eika, C.: On the Mechanism of Platelet Aggregation Induced by Heparin, Protamine and Polybrene. Scandinavian Journal of Haematology, 9:248-257, 1972.

Ellison, N., Ominisky, A.J. and Wollman, H.: Is Protamine a Clinically Important Anticoagulant ? A Negative Answer. Anesthesiology, 35:621-9, 1971.

Ellison, N., Beatty, P.B., Blake, D.R., Wurtzel, H.A. and Mac Vaugh, H.: Heparin Rebound Studies in Patients and Volunteers. The Journal of Thoracic and Cardiovascular Surgery, 67:(5) 723-729, 1974.

Emanuele, R.M.: The Anticoagulant, Antiprotease and Antithrombotic Actions of Various Heparin Fractions Ph.D. Thesis Loyola University, Maywood Illinois, 1987.

Emanuele, R.M., Racanelli, A. and Fareed, J.: The Pharmacokinetics of Different Heparin Molecular Weight Fractions Using 2 Assay Methods. Therapeutic Drug Monitoring, 10:(2) 153-159, 1988.

Enke, A. and Breddin, H.K.: Comparison of a Low Molecular Weight Heparin

and Unfractionated Heparin For The Prevention of Deep Vein Thrombosis in Patients Undergoing Abdominal Surgery. British Journal of Surgery, 75:1058-1063, 1988.

Esposito, R.A., Culliford, A.T., Colvin, S.B., Thomas, S.J., Lackner, L.H. and Spencer, F.C.: The Role of Activated Clotting Time in Heparin Administration and Neutralization for Cardiopulmonary Bypass. Journal of Thoracic and Cardiovascular Surgery, 85:174-185, 1983.

Estes, J.W., Pelikan, E.W and Krueger, E.: A Retrospective Study of the Pharmacokinetics of Heparin. Clinical Pharmacology and Therapeutics, 10:329-337, 1969.

Fabian, I. and Aronson, M.: Polycations as Possible Substitutes for Protamine in Heparin Neutralization. Thrombosis Research, 17:239-247, 1980.

Fabris, F., Fussi, F., Casonato, A., Visentin, L., Randi, M., Smith, M.R. and Girolami, A.: Normal and Low Molecular Weight Heparins:Interactions with Human Platelets. European Journal of Clinical Investigation, 13:135-139, 1983.

Fareed, J., Messmore, H.L. and Bermes, E.W.: New Perspectives in Coagulation Testing. Clinical Chemistry, 26:(10) 1380-91, 1980.

Fareed, J., Messmore, H.L., Walenga, J.M. and Bermes, E.W. Jr.: Synthetic Peptide Substrates in Hemostatic Testing. CRC Clinical Review Clinical Laboratory Science, 19:(2) 71-134, 1983.

Fareed, J., Messmore, H.L., Walenga, J.M. and Bermes, E.W. Jr.: Diagnostic Efficacy of Newer Synthetic Substrate Methods for Assessing Coagulation Variables: A Critical Overview. Clinical Chemistry, 29:(2) 225-236, 1983.

Fareed, J., Walenga, J.M., Hoppensteadt, D.A. and Messmore, H.L.: Studies on the Profibrinolytic Actions of Heparin and Its Fractions. Seminars in Thrombosis and Haemostasis, 11:(2) 199-207, 1985.

Fareed, J., Walenga, J.M., Hoppenstaedt, D.A. and Emanuele, R.M.: Laboratory Studies on the Intravenous and Subcutaneous Administration of PK 10169 in Man. Haemostasis, 16:123-138, 1986.

Fareed, J., Walenga, J.M., Racanelli, A., Hoppensteadt, D., Huan, X., Messmore H.L.: Validity of the Newly Established Low-Molecular-Weight Heparin Standard in Cross-Referencing Low Molecular Weight Heparins. Haemostasis, 3:(18) Suppl. 33-47, 1988.

Fareed, J., Walenga, J.M., Hoppenstaedt, D.A., Huan, X.Q. and Racanelli, A.: Comparative Study on the In Vitro and In Vivo Activities of Seven Low Molecular Weight Heparins. Haemostasis, 3:(Suppl) (3) 3-15, 1988.

Frater, R.W.M., Oka, Y., Hong, Y., Tsubo, T., Loubser, P.G. and Masone, R.: Protamine-Induced Circulatory Changes. Journal of Thoracic and Cardiovascular Surgery, 87:687-692, 1984.

Gans, H.: Study of Mechanisms Which Preserve Blood Fluidity and the Effect of Their Inhibition on Thrombogenesis. Journal of Surgery Research, 6:87-92, 1966.

Gatewood, J.M., Cook, G.R., Balhorn, R., Bradbury, E.M. and Schmid, C.W.: Sequence-Specific Packaging of DNA in Human Sperm Chromatin. Science, 236:962-964, 1988.

Gatti, G., Casu, B., Hamer, G.K. and Perlin, A.S.: Studies on the Conformation of Heparin by ^{1}H and ^{13}C NMR Spectroscopy. Macromolecules, 12:1001, 1979.

Gawoski, J.M.: The Effects of Heparin on the Activated Partial Thromboplastin time of the College of American Pathologists Specimen Survey. Archives of the Pathology Laboratory, 111:785-790, 1987.

Gervin, A.S.: Complications of Heparin Therapy. Surgery, Gynecology and Obstetrics, 140:789-796, 1975.

Gibaldi, M. and Perrier, D.: Pharmacokinetics 2nd ed. Marcel Dekka Inc. New York, 1984.

Godal, H.C. and Gjengedal, G.: Activation of Coagulation by Heparin-Protamine Complexes as Demonstrated by Thrombotest. Scandinavian Journal of Haematology, 8:194-199, 1971.

Gollub, S.: Heparin Rebound in Open Heart Surgery. Surgery, Gynecology and Obstetrics, 3:337-346, 1967.

Goudable, C., Ton That, H., Damani, A., Durand, D., Carnobe, C., Sies, P. and Boneu, B.: Low Molecular Weight Heparin Half Life is Prolonged in Haemodialyzed Patients. Thrombosis Research, 43:1-5, 1986.

Graham, D.T., Pomeroy, A.R. and Smythe, D.B.: Measurement of the Heparin Neutralizing Capacity of Protamine. Thrombosis and Hemostasis (Stuttg), 41:583-589, 1979.

Gram, J., Mercker, S., and Bruhn, H.D.: Does Protamine Chloride Neutralize Low Molecular Weight Heparin Sufficiently? Thrombosis Research, 52:353-359, 1988.

Guarini, S. and Ferrari, W.: Sodium Deoxycholate Promotes the Absorption of Heparin Administered Orally, Probably by Acting on Gastrointestinal Mucosa in Rats. Experientia, 41:350-352, 1985.

Gusse, M., Sautiere, P., Chauviere, M. and Chevaillier, P.: Extraction, Purification and Characterization of the Sperm Protamines of the Dog-Fish Scylliorhinus Caniculus. Biochemie and Biophysica Acta, 748:93-98, 1983.

Guven, K.C., Arabacioglu, F., Atkin, E., Ulitin, S.B. and Koyuncuoglu, H.: The Lipolytic, Anticoagulant and Fibrinolytic Activities of Heparin Fractions. Journal of the Pharmaceutical Industry, 36:201-208, 1974.

Han, P. and Ardle, N.G.: Heparin Platelets and Blood Coagulation:Implications For Low Doses of Heparin Prophylactic Regiments in Venous Thrombosis. British Journal of Haematology, 27:253-272, 1974.

Harenberg, J., Wurzner, B., Zimmermann, R. and Schettler, G.: Bioavailability and Antagonism of the Low Molecular Weight Heparin CY 216 in Man. Thrombosis Research, 44:549-554, 1986.

Harenberg, J., Gnasso, A., de Vries, J.X., Zimmermann, R. and Augustin, J.: Inhibition of Low Molecular Weight Heparin by Protamine Chloride In Vivo. Thrombosis Research 38:11-20, 1985.

Harenberg, J., Wurzner, B., Schetter, G., and Zimmerman, R. Normal and Low Molecular Weight Heparin Prolong Bleeding Time. Thrombosis and Haemostasis, 54 (1) 30, 1985.

Horner A.A.: Enzymatic Depolymerization of Macromolecular Heparin as a Factor in Control of Lipoprotein Lipase Activity. Proceedings of the National Academy of Science, 69:3469, 1972.

Hepatitis B Elisa Test, Product Insert, Abbott Laboratories.

Heptinstal, S. and Graham, P.M.: Adenosine Diphosphate Induced Platelet Aggregation and Release Reaction in Heparinized Platelet Rich Plasma and the Influence of Added Citrate. British Journal of Haematology, 36:566-571, 19797.

Hirsh, J. and Brain, E.A.: Hemostasis and Thrombosis. A Conceptual Approach. Churchill Livingstone, New York, 1983.

HIV Product Insert, Abbott Labs, February 1987.

Hobbelen, P.M.J., Vogel, G.M.T. and Meuleman, D.G.: Pharmacokinetic Behaviour of Antithrombotic and Bleeding Enhancing Effects of Various Heparin(oid)s in Rats: Their Relation with Interactions with Factors IIa and Xa. Thrombosis and Hemostasis, 54 (1), 1-372, 1985.

Holmer, E. and Soderstrom, G.: Neutralization of Unfractionated Heparin and a Low Molecular Weight Heparin Fragment by Protamine. Thrombosis and Haemostasis, 50:103, 1983.

Hook, M., Bjork, I., Hopwood, J., and Lindahl, U.: Anticoagulant Activity of Heparin:Separation of High Activity and Low Activity Heparin Species by Affinity Chromatography on Immobilized Antithrombin. Febs Letters, 66:90-93, 1976.

Hoppensteadt, D.A., Walenga, J.M. and Fareed, J.: Validity of Serine Protease Inhibition Tests in the Evaluation and Monitoring of the Effect of Heparin and Its Fractions. Seminars in Thrombosis and Hemostasis, 11:(2) 112-120, 1985.

Horellou, M.H., Conrad, J., Lecrubier, C., Samama, M., Roque-D'Orbcastel, de Fenoyl, O., Di Maria, G. and Bernadou, A.: Persistent Heparin Induced Thrombocytopenia Despite Therapy with Low Molecular Weight Heparin. Thrombosis and Haemostasis, 51:(1) 134, 1984.

Horrow, J.C.: Protamine:A Review of Its Toxicity. Anesthesia and Analgesia, 64:348-361, 1985.

Hougie, C.: Anticoagulant Action of Protamine Sulfate. Proceedings of the Society of Experimental Biology in Medicine, 98:130-133, 1958.

Howell, W.H.: Heparin an Anticoagulant. American Journal of Physiology, 63:434-435, 1922-23.

Huang T.H., Ordu, Y., Demple, G., Stehle, D.L., Heene Harenberg, J.:Binding of Heparins to Protamine. Thrombosis and Hemostasis, 62:(1) 514, 1989.

Hubbard, A.R. and Jennings, C.A.: Neutralisation of Heparan Sulphate and Low Molecular Weight Heparin by Protamine. Thrombosis and Hemostasis, 53:(1) 86-89, 1985.

Hurst, R.E., Menter, J., West, G., Settine, J. and Coyne, E. Structural Basis for the Anticoagulant Activity of Heparin. Relationship to the Number of Charged Groups. Biochemistry, 18:4283-4287, 1979.

Hurst, R.E., Van Dedem, G., and Settine, J.: Biophysical Characteristics of Anionic Density Fractionated Mucosal Heparin in Relation to Potencies in Anticoagulant and Thrombin Inhibition Assays. Thrombosis Research, 22:633-643, 1981.

Hurst, R.E., Poon, M-C. and Griffith, M.J.: Structure Activity Relationship of Heparin. Independence of Heparin Charge Density and Antithrombin Binding Domains in Thrombin Inhibition by Antithrombin and Heparin and Heparin Cofactor II. Journal of Clinical Investigation, 72:1042-1045, 1983.

Ingles, C.J., Trevithick, J.R., Smith, M., and Dixon, G.H.: Biosynthesis of Protamine During Spermatogenesis in Salmonoid Fish. Biochemical and Biophysical Research Communications, 22:627-34, 1966.

Israels, E.D.: Partial Thromboplastin Time in the Presence of Heparin. American Journal of Clinical Pathology, 77:(3), 321-324, 1988.

Jacobson, I., Backstrom, E., Hook, M. and Lindahl, U.: Biosynthesis of Heparin:Assay and Properties of the Microsomal Uronosyl C-5 Epimerase. Journal of Biological Chemistry, 254:2975, 1979.

Jaques, L.B., Charles, A.F. and Best, C.H.: The Administration of Heparin. Acta Med Scand, 90:(Suppl) 190, 1938.

Jaques, L.B.: A Study of the Toxicity of the Protamine, Salmine. British Journal of Pharmacology, 4:135-144, 1949.

Jaques, L.B.: Protamine-Antagonist to Heparin. Journal of Canadian Medical Association, 108:1291-1297, 1973.

Jaques, L.B., Mahadoo, J. and Kavanagh, L.W.: Intrapulmonary Heparin: A New Procedure for Anticoagulant Therapy. Lancet ii:1157, 1976.

Jaques, L.B. and Mahadoo, J.: Pharmacodynamics and Clinical Effectiveness of Heparin. Seminars in Thrombosis and Hemostasis 4:(4) 298-325, 1978.

Jones, G.R., Hashim, R. and Power, D.M.: A Comparison of the Strength of Binding of Antithrombin III, Protamine and Poly-L-Lysine to Heparin Samples of Different Anticoagulant Activities. Biochimica et Biophysica Acta, 883:69-76, 1986.

Jorpes, E.: The Chemistry of Heparin. Biochemical Journal, 29:1817-1830, 1935.

Kakkar, V.V., Stamatakis, J.D., Bentley, P.G., Lawrence, D., DeHaas, H.A. and Ward, V.P.: Prophylaxis For Postoperative Deep Vein Thrombosis. Synergistic Effect of Heparin and Dihydroergotamine. Journal of the American Medical Association, 241,39-42, 1979.

Kakkar, V.V. and Murray, W.J.G.: Efficacy and Safety of Low Molecular Weight Heparin (CY 216) in Preventing Postoperative Venous Thromboembolism: A Cooperative Study. British Journal of Surgery, 72:786-791, 1985.

Kelton, J.G.: Heparin-Induced Thrombocytopenia. Haemostasis 16:173-186, 1986.

Kesteven, P.J., Ahmed, A., Aps, C., Williams, B.T. and Savidge, G.F.: Protamine Sulfate and Heparin Rebound Following Open Heart Surgery. Journal of Cardiovascular Surgery, 27:600-603, 1986.

Kim, T.D., Sakon, T., Kawasaki, T., Oshiro, T. and Mori, T.: Studies on Liposome-Encapsulated Heparin. Thrombosis Research, 43:603-612, 1986.

King, F.A., Tarbrough, C.J., Anderson, D.C., Gordon, T.P. and Gould, K.G.: Primates. Science, 240:(4858) 1475-1482, 1988.

Kirklin, J.K., Chenoweth, D.E., Naftel, D.C., Blackstone, E.H., Kirklin W., Bitran D.D., Curd J.G., Reves J.G. and Samuelson P.N. Effects of Protamine Administration after Cardiopulmonary Bypass on Complement, Blood Elements, and the Hemodynamic State. Annals of Thoracic Surgery, 41:193-199, 1986.

Kitani, T., Nagarajan, S.C. and Shanberge, J.N. Effect of Protamine on Heparin-Antithrombin III Complexes. In Vivo Studies. Thrombosis Research 17:375-382, 1980.

Kitani, T., Nagarajan, S.C. and Shanberge, J.N.: Effect of Protamine on Heparin-Antithrombin III Complexes. In Vitro Studies. Thrombosis Research 17:367-374, 1980.

Kjellen, L.: Biosynthesis of Heparin. The Medical Journal of Australia, 144:Hs 3-6, 1986.

Koller, M., Schoh, U. and Buchmann W.: Low Molecular Weight Heparin (Kabi 2165) as Thromboprophylaxis in Elective Visceral Surgery. Thrombosis and Haemostasis 56:243-246, 1986.

Kossel, A.: The Protamines and Histones, Longman Green and Co., London and New York, 1928.

Krauss, R.M., Levy R.I. and Fredrickson, D.S.: Selective Measurement of Two Lipase Activities in Post Heparin Plasma from Normal Subjects and Patients with Hypoproteinemia. Journal of Clinical Investigation, 54:1107-1124, 1974.

Lam, L., Silbert, J.E. and Rosenberg, R.D. The Separation of Active and Inactive Forms of Heparin. Biochemical and Biophysical Research Communications, 69:570-577, 1976.

Lane, D.A., Macgregor, I.R., Michalski, R. and Kakkar, V.V.: Anticoagulant Activities of Four Unfractionated and Fractionated Heparins. Thrombosis Research, 12:257-271, 1978.

Lane, D.A., Flynn, R., Pejler, G., Lindahl, U., Choay, J. and Preissner, K.: Structural Requirements for the Neutralization of Heparin-like Saccharides by Complement S Protein/Vitronectin. Journal of Biological Chemistry, 262:(34) 16343-16348, 1987.

Larsen, A.K., Hetelekidis, S. and Langer, R.: Disposition and Anticoagulant Activity of Biologically Active Heparin Fragments in the Rat. Journal of Pharmacology and Experimental Therapeutics, 231:(2), 373-8, 1984.

Leyvraz, P.F., Richard, J., Bachmann, F., Van Melle, J.M., Treyvaud, J.J. Livio J. and Candardjis, G.: Adjusted Versus Fixed-Dose Subcutaneous Heparin in the Prevention of Deep Venous Thrombosis AFter Total Hip Replacement. New England Journal of Medicine, 309:954-958, 1983.

Lijnen, H.R., van Hoeff, B. and Collen D.: Interaction of Heparin with Histidine-Rich Glycoprotein and with Antithrombin III. Thrombosis and Haemostasis, 50:(2) 560-562, 1983.

Lindahl, U., Hook, M. and Backstrom, G.: Structure and Biosynthesis of Heparinlike Polysaccharides. Federation Proceedings, 36:19, 1977.

Lindahl, U. and Bjork, I.: Basic Mechanism of Action of Heparin. (abstr.) Thrombosis and Haemostasis, 50:265, 1983.

Lindblad, B., Borgstrom, A., Wakefield, T.W., Whitehouse, W.M. and Stanley, J.: Protamine Reversal of Anticoagulation Achieved with a Low Molecular Weight Heparin. The Effects on Eicosanoids, Clotting and Complement Factors. Thrombosis Research 48:31-40, 1987.

Ling, V., Trevithick, J.R., Dixon, G.H.: The Biosynthesis of Protamine in Trout Testis. I. Intracellular Site of Synthesis. Canadian Journal of Biochemistry, 47:51-60, 1969.

Ling, V., Jergil, B., and Dixon, G.: The Biosynthesis of Protamine in Trout Testis. Journal of Biological Chemistry, 246:1168-1176, 1971.

Linker, A. and Hovingh, P.: Enzymatic Degradation of Heparin as a Tool for Structural Analysis. In Heparin: Structure, Cellular Functions and Clinical Implications. (McDuffie N. Ed) Academic Press, New York, 3-24, 1977.

Liu, C.Y., Scott, C.F. and Bagdasarian, A.: Potentation of the Function of Hageman Factor Fragments by HMW Kininogen. Journal of Clinical Investigation, 60:7, 1977.

Lockner, D., Bratt, G., Tornebohn, E. and Aberg, W.: Pharmacokinetics of Intravenously and Subcutaneously Administered Fragmin in Healthy Volunteers. Haemostasis, 16:(suppl.) 2, 8-10, 1986.

Lormeau, J.C.: Physiochemical and Biological Characteristics of CY 216. Fraxiparine First International Symposium. Analytical and Structural Data, Pharmacology, Clinical Trials. Schattauer New York, 1989.

Losito, R., Gattiker, H. and Bilodeau, G.: Gel Chromatography of Heparin. Journal of Chromatography, 226:61-67, 1981.

Lowary, L.R., Smith, F.A. and Coyne, E.: Comparative Neutralization of Lung and Mucosal Derived Heparin and Protamine Sulfate Using In Vitro and In Vivo Methods. Journal of Pharmaceutical Sciences, 60:638, 1971.

Lowenstein, E., Johnston, W.E., Lappas, D.G., D'Ambra, N.D., Schneider, R.C., Daggett, W.M., Akins, C.W. and Philbin, D.M.: Catastrophic Pulmonary Vasoconstriction Associated with Protamine Reversal of Heparin. Anesthesiology, 59:470-473, 1983.

Mahadoo, J.: Pulmonary Absorption of Heparin. Ph.D. Thesis University of Saskatchewan, Sask. Canada, 1978.

Mahadoo, J. and Jaques, L.B.: Cellular Control of Heparin in Blood. Medical Hypothesis, 5:835-841, 1979.

Man-Chiu Poon, Hurst, R.E. and Rives, M.S.: Platelet Factor Four and Protamine Sulfate Neutralization of Heparin According to Anionic Charge Density. Thrombosis and Haemostasis, 47:(2) 162-165, 1982.

Marchisio, M.A., Sbertoli, C., Farina, G. and Ferruti, P.: A New Antagonist of Heparin-Partially N-Oxidized Polyallyldiethylamine. European Journal of Pharmacology, 12:236-242, 1970.

Mardiguian, J. and Trillou, M.: Studies on Heparin Fragments: Relationship Between the Degree of Polymerization and the Factor Xa and Thrombin Inhibition Activity (abstract). Thrombosis and Haemostasis, 50:695, 1983.

Markwardt, F. and Klocking, H.P.: Heparin Induced Release of Plasminogen Activator. Haemostasis, 6:370-374, 1977.

Marushige, K., Ling, V., Dixon, G.H.: Phosphorylation of chromosomal basic proteins in maturing trout testis. Journal of Biological Chemistry, 244: 5953-5958, 1969.

Massonnet-Castel, S., Pelissier, E., Bara, L., Terier, E., Abry, B., Guibourt, P., Swanson, J., Jaulmes, B., Carpentier, A., and Samama, M.: Partial Reversal of Low Molecular Weight Heparin (PK 10169) Anti Xa Activity by Protamine Sulfate:In Vitro and In Vivo Study During Cardiac Surgery with Extracorporeal Circulation. Haemostasis, 16:139-146, 1986.

Mastacchi, R. and Barbati, M.: Inactivation of the Heparin Anticoagulant Activity by Rat Kidney Lysosmal Enzymes. Thrombosis Research, 47:101-106, 1987.

Mattson, C., Hoylaerts, M., Holmer, E., Uthene, T. and Collen, D.: Antithrombotic Properties in Rabbits of Heparin and Heparin Fragments Covalently Coupled to Human Antithrombin III. Journal of Clinical Investigation, 75:1169-1173, 1985.

Mayer, P.R. and Brazzell, R.K.: Application of Statistical Moment Theory to Pharmacokinetics. Clinical Pharmacology, 28:481-483, 1988.

Mc Farlane, D.E., Walsh, P.N., Mills, D.C., Holmes, H. and Day, H.J.: The Role of Thrombin in ADP Induced Platelet Aggregation and Release:A Clinical Evaluation. British Journal of Haematology, 30:453-461, 1975.

Mc Farlane, R.G.: An Enzyme in the Blood Clotting Mechanism and its Function as a Biochemical Amplifier. Nature, 202:498-499, 1964.

Mc Duffie, N.M., Dietrich, C.P. and Nader, H.B.: Electrofocussing of Heparin: Fractionation of Heparin Into 21 Components Distinguishable From Other Acidic Mucopolysaccharides. Biopolymers, 14:1473-1486, 1975.

McLean, J.: The Thromboplastic Action of Cephalin. American Journal of Physiology, 41:250-257, 1916.

Meischer F.: Das Protamin, Elne Reue Organische. Base Aus Den Samenfaden Des Rheinlaschses, Ber, 7:376-379, 1874.

Merton, R.E., Thomas, D.P., Havercroft, S.J., Barrowcliffe, T.W. and Lindahl, U.: High and Low Affinity Heparin Compared with Unfractionated Heparin as Antithrombotic Drugs. Thrombosis and Haemostasis, 51:254-256, 1984.

Messmore, H.L., Fareed, J., Parvez, Z.F., Zuckerman, L. and Squillaci, G.: Heparin Induced Thrombocytopenia. Can Low Molecular Weight Heparin Be Substituted Safely? (Abstract). Thrombosis and Haemostasis, 50:55, 1983.

Messmore, H.L.: Clinical Use of Heparin Fractions, Fragments and Heparinoids. Seminars in Thrombosis and Hemostasis, 11:(2) 208-12, 1985.

Messmore, H.L.: Clinical Efficacy of Heparin Fractions:Issues and Answers. CRC Critical Revue Clinical Laboratory, 23:(2), 77-94, 1986.

Michalski, R., Lane, D.A., Pepper, D.S. and Kakkar, V.V.: Neutralization of Heparin in Plasma By Platelet Factor 4 and Protamine Sulphate. British Journal of Haematology, 38:561-571, 1978.

Monkhouse, F.C., Dickie, H.: Physiological Factors Concerned with the Removal of Injected Heparin from Circulating Blood. American Journal of Physiology: 178: 223, 1954.

Nader, H.B., McDuffie, H.M. and Dietrich, C.P.: Heparin Fractionation by Electrofocussing:Presence of 21 Components of Different Molecular Weights. Biochemical Research Communications, 57:488-493, 1974.

Neufield, E.F., Lim, T.W. and Shapior, L.J.: Inherited Disorders of Lysosomal Metabolism. Annual Review of Biochemistry, 44:357, 1975.
Nilsson-Ehle, P., Garfinkel, A.S., Schotz, M.C.: Lipolytic Enzymes and Plasma Lipoprotein Metabolism. Annual Revue of Biochemistry, 49:667-693, 1980.

O'Brien, J.R., Shoobridge, S.M. and Finch, W.J.: Comparison of the Effect of Heparin and Citrate on Platelet Aggregation. Journal of Clinical Pathology, 22: 28-35, 1969.

O'Brien, J.R., Etherington, M.D. and Pashley, M.: Intra-Platelet Factor 4 (IP.PF4) and the Heparin-Mobilisable Pool of PF4 in Health and Atherosclerosis. Thrombosis and Haemostasis, 51:(3) 354- 357, 1984.

Ockelford, P.A., Carter, C.J., Ceerskus, A., Smith, C.A. and Hirsh, J.: Comparison of the In Vivo Hemorrhagic and Antithrombotic Effects of a Low Antithrombin III Affinity Heparin Fraction. Thrombosis Research, 27:679-690, 1982.

Ofosu, F.A., Blajchman, M.A., Modi, G.J., Smith, L.M., Buchanan, M.R. and Hirsh, J.: The Importance of Thrombin Inhibition for the Expression of the Anticoagulant Activities of Heparin, Dermatan Sulphate, Low Molecular

Weight Heparin and Pentosan Polysulphate. British Journal of Haematology, 60:695-704, 1985.

Piper, J.: The Fate of Heparin in Rabbits After Intravenous Injection. Filtration and Tubular Secretion in the Kidneys. Acta Pharmacolica et Toxicologica, 3:373-384, 1947.

Ofosu, F., Blajchamn, M.A., Modi, G., Cerskus, A.L. and Hirsh, J.: Activation of Factor Xa and Prothrombin in Antithrombin III Depleted Plasma:The Effects of Heparin. Thrombosis Research, 23:331-345, 1981.

Oh, T.H., Naidoo, S.S. and Jaques, L.B.: The Uptake and Disposition of ^{35}SHeparin by Macrophages In Vitro. Journal of Reticuloendothelial Society, 13: 134, 1973.

Okajima, Y., Kanayama, S., Maeda, Y., Urano, S., Kitani, T., Watada, M., Nakagawa, M., and Ijichi, H.: Studies on the Neutralizing Mechanism of Antithrombin Activity of Heparin by Protamine. Thrombosis Research, 24:21-29, 1981.

Oosta, G.M., Gardner, W.T., Beeler, D.L. and Rosenberg, R.D.: Multiple Functional Domains of the Heparin Molecule. Proceedings of the National Academy of Science, 78:829-833, 1981.

Oosta, G.M., Favreau, L.V., Beeler, D.L.and Rosenberg R.D.: Purification and Properties of Human Platelet Heparitinase. Journal of Biological Chemistry, 257:11249, 1982.

Oppenheimer, J.H., Schwartz, H.L. and Surks, M.I.: Determination of Common Parameters of Iodothyronine Metabolism and Distribution in Man: Noncompartmental Analysis, Journal of Clinical Endocrinology and Metabolism, 41:319- 324, 1975.

Oshima, G.: Prevention of Thrombin/Antithrombin III Reaction in the Presence of Protamine or Polybrene. Thrombosis Research, 51:566-574, 1988.

Palm M. and Mattsson Ch. Pharmacokinetics of Heparin and Low Molecular Weight Heparin Fragment (Fragmin) in Rabbits with Impaired Renal or Metabolic Clearance. Thrombosis and Haemostasis, 58:(3) 932-935, 1987.

Paques, E.P., Stohr, H.A. and Heimburger, N.: Study on the Mechanism of Action of Heparin and Related Substances of the Fibrinolytic System: Relationship Between Plasminogen Activators and Heparin. Thrombosis Research, 42:797-807, 1986.

Perl, W. and Samuel, P.: Input-Analysis for Total Input Rate and Total Traced Mass of Body Cholesterol in Man. Circulation Research, 25:191-199, 1969.

Persson, E., Nordenstrom, J. and Nilsson-Ehle, P.: Plasma Kinetics of Lipoprotein Lipase and Hepatic Lipase Activities Induced by Heparin and

a Low Molecular Weight Heparin Fragment. Scandinavian Journal of Clinical Laboratory Investigation, 47:151-155, 1987.

Persson, E., Nordenstrom, J., Hagenfeldt, L. and Nilsson-Ehle, P.: Plasma Lipolytic Activity After Subcutaneous Administration Of Heparin and a Low Molecular Weight Heparin Fragment. Thrombosis Research, 46:697-704, 1987.

Persson, E., Nordenstrom, J., Nilsson-Ehle, P.F., and Hagenfeldt, L.: Lipolytic and Anticoagulant Activities of a Low Molecular Weight Fragment of Heparin. European Journal of Clinical Investigation, 15:215-220, 1985.

Physicians Desk Reference 1989, Medical Economics Company Inc., Oradell N.J., p.2166.

Piffarre, R., Babka, R., Sullivan, H., Montoya, A., Bahkos, M. and El-Etr, A.: Management of Postoperative Heparin Rebound Following Cardiopulmonary Bypass. Journal of Thoracic and Cardiovascular Surgery, 81:378-381, 1981.

Planes, A., Vochelle, N., Mazas, F., Mansat, C., Zucman, J., Landais, A., Pascariello, J.C., Weill, D. and Butel, J.: Prevention of Postoperative Venous Thrombosis:A Randomized Trial Comparing Unfractionated Heparin With Low Molecular Weight Heparin In Patients Undergoing Total Hip Replacement. Thrombosis and Haemostasis, 60 407-410, 1988.

Racanelli, A., Hoppensteadt, D., and Fareed, J.: In Vitro Protamine Neutralization Profiles of Heparins Differing in Source and Molecular Weight. Seminars in Thrombosis and Hemostasis (in press).

Racanelli, A., Breddin, H.K., Krupinski, K. and Fareed, J.: Antagonism of the Antithrombotic Effects of Heparin and Fraxiparine^{R} in a Rat Model of Laser Induced Thrombosis. Structure and Activities of Heparin and Related Mucopolysaccharides. Annals of the New York Academy of Sciences 566:473-475, 1989.

Racanelli, A., Fareed, J., Huan, X.Q.: Low Molecular Weight Heparin-Induced Bleeding Can Be Neutralized by Protamine. Haemostasis, 18: (Suppl 2), 163-164, 1988.

Racanelli, A., Fareed, J., Walenga, J.M. and Coyne, E.: Biochemical and Pharmacologic Studies of the Protamine Interactions with Heparin, Its Fractions and Fragments. Seminars in Thrombosis and Hemostasis, 11:(2), 176-189, 1985.

Rappaport, R.S., Blume, M.R. and Hung, P.P.: Endothelial Cell Growth Factors and Heparin Stimulate the Synthesis of Tissue-Type Plasminogen Activator and Prourokinase by Human Diploid Fibroblasts (abstract). Fibrinolysis, Suppl 1:154,1988.

Ratnoff, O.D., Davie, E.W., Mallet, S.L.: Studies On The Action of Hageman Factor:Evidence That Activated Hageman Factor In Turn Activates Plasma Thromboplastin Antecedent. Journal of Clinical Investigation, 40:803, 1961.

Robinson, H.C., Horner, A.A., Hook, M., Ogre S. and Lindahl, U.: A Proteoglycan Form of Heparin and its Degradation to Single Chain Molecules. Journal of Biological Chemistry, 253:6687, 1978.

Rodemer, B., Hellstern, P., Wetzler, E. and Wenzel, E.: Plasma Heparin-Like Activities After Percutaneous Application of a Low Molecular Weight Heparin in Healthy Subjects. Thrombosis and Hemostasis, 56:(1) 109, 1986.

Roden, L., and Horowitz, M.I.: Structure and Biosynthesis of Connective Tissue Proteoglycans. In Horowitz M.I., Pigman W.(eds) The Glycoconjugates, Vol II p.3. New York, Academic Press, 1977.

Rosenberg, J.S., McKenna, P.W. and Rosenberg, R.D.: Inhibition of Human Factor IXa By Human Antithrombin. Journal of Biological Chemistry, 250:(23) 8883-8888, 1975.

Sagher F. and Even-Paz, Z.: Mastocytosis and the Mast Cell p. 282 (Karger) Phiebig, 1967.

Salzmann, E.W., Deykin, D., Shapiro, R.M. and Rosenberg, R.D.: Management of Heparin Therapy. Controlled Prospective Trial. New England Journal of Medicine, 292, 1046-1050, 1975.

Salzman, E., Rosenberg, R.D., Smitt, M.H., Lindon, J.N. and Favreau, L.: Effect of Heparin and Heparin Fractions on Platelet Aggregation. Journal of Clinical Investigation, 65:64-73, 1980.

Salzman, E.W.: Low Molecular Weight Heparin. Is Small Beautiful? New England Journal of Medicine, 315:(15), 957-959, 1986.

Samama, M.: Les Nouvelles Heparines. Presse Med., (15), 1631-1635, 1986.

Samama, M., Boissel, J.P., Combe-Tamzali, S. and Leizorovicz, A.: Clinical Studies with Low Molecular Weight Heparins in the Prevention and Treatment of Venous Thromboembolism. Structure and Activities of Heparin and Related Mucopolysaccharides. Annals of the New York Academy of Sciences 566:386-398, 1989.

Schalm, O.W., Jain, N.C. and Carroll, E.J.: Veterninary Hematology, Lea and Febiger, Philadelphia, 1975.

Schiele, U. and Heuk, H.C.: The Effect of Polycations on the Inhibitory Action of Antithrombin III in the Absence and Presence of Polyanions. Thrombosis Research, 43:187-193, 1986.

Schmitz-Huebner, U., Bunte, H. and Friese, G.: Clinical Efficacy of Low Molecular Weight Heparin in Postoperative Thrombosis Prophylaxis. Klin Wochenschr, 62:349-353, 1984.

Schwartz, L.B. and Bradford, T.R.: Regulation of Tryptase From Human Mast Cells By Heparin. Stabilization of the Active Tetramer. Journal of Biological Chemistry, 261:7372-7379, 1986.

Shanberge, J.N., Kambayashi, J. and Nakagawa, M.: The Interaction of Platelets with a Tritium Labelled Heparin. Thrombosis Research, 9:595-609, 1976.

Shanberge, J.N. and Sridhar, C.N.: The Interaction of Protein Bound Heparin and Antithrombin III. Annals of the New York Academy of Sciences, 370:708-13, 1981.

Shanberge, J.N., Murato, M., Quattrociocchi-Longe T.M. and Van Nest L.: Heparin-Protamine Complexes in the Production of Heparin Rebound and Other Complications of Extracorporeal Bypass Procedures. American Journal of Clinical Pathology, 87: 210-217, 1986.

Shanberge, J.N., Quattrociocchi, T.M. and Martens M.H.: Interrelationship of Protamine and Platelet Factor 4 in the Neutralization of Heparin. Thrombosis Research, 46:89-100, 1987.

Sie, P., Cremers, B., Dupouy, D., Caranobe, C., Dol, F. and Boneu, B.: Neutralization of Dermatan Sulfate In Vitro and In Vivo by Protamine and Polybrene. Thrombosis Research, 54:(1) 63-74, 1989.

Silbert, J.E.: Biosynthesis of Heparin Formation of a Sulfated Glycosaminoglycan with a Microsomal Preparation from Mast Cells Tumors. Journal of Biological Chemistry, 242:5146, 1967.

Stanzani, L., Mascellani, G., Corbelli, G.P. and Bianchini, P.: Rectal Absorption of Some Glycosaminoglycan Sulfates and Heparin in Rats. Pharmaceutical Pharmacology, 33:783-786, 1981.

Stassen, J.M., Juhan-Vague, I., Alessi, M.C., DeCock, F. and Collen, D.: Potentiation by Heparin Fractions of Thrombolysis Induced with Human Tissue-Type Plasminogen Activator or Human Single-Chain Urokinase-Type Plasminogen Activator. Thrombosis and Haemostasis, 58:(3) 947-950, 1987.

Stewert, W.J., McSweeney, S.M., Kellett, M.A., Kellet, M.D., Faxon, D.P. and Thomas, J.R.: Increased Risk of Severe Protamine Reactions in NPH Insulin-Dependant Diabetics Undergoing Cardiac Catheterization. Circulation, 70:(5) 788-792, 1984.

Stoelting, R.K.: Allergic Reactions During Anesthesia. Anesthesia and Analgesia, 62:342-56, 1983.

Sue, T.K.: Heparin: Structure, Cellular Functions and Clinical Applications. Academic Press, Inc. N.Y., 1979.

Tan, F., Jackman, H., Skidgel, R.A., Zsigmond, E. and Erdos, E.: Protamine Inhibits Plasma Carboxypeptidase N, the Inactivator of Anaphylatoxins and Kinins. Anesthesiology, 70:267-275, 1989.

Teien, A.M.: Heparin Elimination in Patients with Liver Cirrhosis. Thrombosis and Haemostasis, 11:(2) 107-117, 1977.

Thomas, D.P.: Annotation:Heparin, Low Molecular Weight Heparin and Heparin Analogues. British Journal of Haematology, 58:385-390, 1984.

Thomas, D.P.: Current Status of Low Molecular Weight Heparin. Thrombosis and Haemostasis, 3:241-242, 1986.

Thompson, C.C., Forbes, C.D., Prentice, C.R.M.: The Potentiation of Platelet Aggregation by Heparin In Vivo and In Vitro. Clinical Science and Molecular Medicine, 45:485-494, 1973.

Thompson, W.H.: Die Physiologische Wirkung Der Protamine und Ihrer Spaltungspropukle. Journal of Physiological Chemistry, 29:1, 1900.

Tobita, T., Minoru, N., Minoru, N. and Ando, T.: Isolation and Characterization of Nuclear Basic Protein (Protamine) from Boar Spermatozoa. Biochimica et Biophysica Acta, 707:252-258, 1982.

Turpie, A.G.G., Levine, M.N. and Hirsh J.: A Randomized Controlled Trial of PK 10169, Low Molecular Weight Heparin In the Prevention of Deep Vein Thrombosis in Patients Undergoing Elective Hip Surgery. Klin Wochenschr, 62:349-353, 1984.

Turpie, A.G.G., Levine, M.N., Hirsh, J., Carter, C.J., Jay, R.M., Powers, M., Andrew, R.D., Hull, R. and Gent, M.: A Randomized Controlled Trial of a Low Molecular WEight Heparin (Enoxaparine) to Prevent Deep-Vein Thrombosis in Patients Undergoing elective Hip Surgery. New England Journal of Medicine, 315 925-929, 1986.

Ueno, M., Nakasaki, T., Horikoshi, I. and Sakuragawa, N.: Oral Administration of Liposomally-Entrapped Heparin to Beagle Dogs. Chemical Pharmacy Bulletin, 30: 2245-2247, 1982.

Umlas, J., Taff, R.H., Gauvin, G. and Swierk, P.: Anticoagulant Monitoring and Neutralization During Open Heart Surgery-A Rapid Method for Measuring Heparin and Calculating Safe Reduced Protamine Doses. Anesthesiology and Analgesia, 62: 1095-109, 1983.

Unvas, B.: The Molecular Basis for the Storage and Release of Histamine in Rat Mast Cell Granules. Life Sciences, 14:2355-2366, 1974.

Vairel, E.G., Bouty-Boye, H., Toulemonde, F., Doutremepuich, C., Marsh, N.A., and Gaffney, P.J.: Heparin and a Low Molecular Weight Fraction Enhances Thrombolysis and this Pathway Exercises a Protective Effect Against Thrombosis. Thrombosis Research, 30:219-224, 1983.

Vinazzer, H., Stemberger, A., Haas, S. and Blumel, G.: Influence of Heparin: Of Different Heparin Fractions and of Low Molecular Weight Heparin-Like Substances on the Mechanism of Fibrinolysis. Thrombosis Research, 27:341-352, 1982.

Virchow, R.: Phlogose and Thrombose in Gefassystem. Gesammelte Abhandlungen zur Wissenschafteeichen Medicin: Frankfurt, Von Meidinger Sohn, 458-638, 1856.

Von Kaulla, K.N. and McDonald, T.S.: The Effect of Heparin on Components of the Human Fibrinolytic System. Blood 13:811-821, 1958.

Wakefield, T.W., Hantler, C.B., Lindblad, B., Whitehouse, W.M. and Stanley, J.C.: Protamine Pretreatmant Attenuation of Hemodynamic and Hematologic Effects of Heparin-Protamine Interaction. Journal of Vascular Surgery, 3:(6) 885-888, 1986.

Walenga, J.M.: Factor Xa Inhibition In Mediating Antithrombotic Actions: Application of A Synthetic Pentasaccharide. Ph.D Thesis. The University Pierre et Marie Curie Paris VI, 1987.

Walenga, J.M., Petitou, M., Lormeau, J.C., Samama, M., Fareed, J. and Choay, J.: Antithrombotic Activity of a Synthetic Heparin Pentasaccharide in a Rabbit Stasis Thrombosis Model Using Different Thrombogenic Challenges. Thrombosis Research, 46:187-198, 1987.

Walenga, J.M., Fareed, J., Hoppensteadt, D. and Emanuele, R.M.: In Vitro Evaluation of Heparin Fractions : Old vs. New Methods. CRC Critical Revues: Clinical Laboratory Science, 22:361-369, 1986.

Weichert, W., Pauliks, J., and Breddin, H.K.: Laser-Induced Thrombi in Rat Mesenteric Vessels and Antithrombotic Drugs. Haemostasis, 13:61-71, 1983.

Weichert, W., Grun, H., Muther, B., Steinhoff, W., Breddin, H.K.: Effect of Prostaglandins on Platelet Function and Laser-Induced Thrombus Formation in Rat Mesenteric Arterioles. Schor (ed). Prostaglandins and Other Eicosanoids in the Cardiovascular System, Proc. 2nd Int Symposium Nurnberg-Furth 303-309, 1984.

Weichert, W. and Breddin, H.K.: Laser-Induced Thrombosis in the Microcirculation of Rat Mesenteric Vessels. Biology and Pathology of Platelet-Vessel Wall Interactions, Academic Press,1986.

Wessler, S. Small Doses of Heparin and a New Concept of Hypercoagulability. Thrombosis and Haemostasis, 33:81-86, 1974.

Westwick, J., Scully, M.F., Poll, C. and Kakkar, V.V.: Comparison of the Effects of Low Molecular Weight Heparin and Unfractionated Heparin on Activation of Human Platelets In Vitro. Thrombosis Research, 42:435-447, 1986.

Yin, E.T., Wessler, S., Butler, J.V.: Plasma Heparin: A Unique, Practical, Submicrogram-sensitive assay. Journal of Laboratory Clinical Medicine, 2:298-310, 1973.

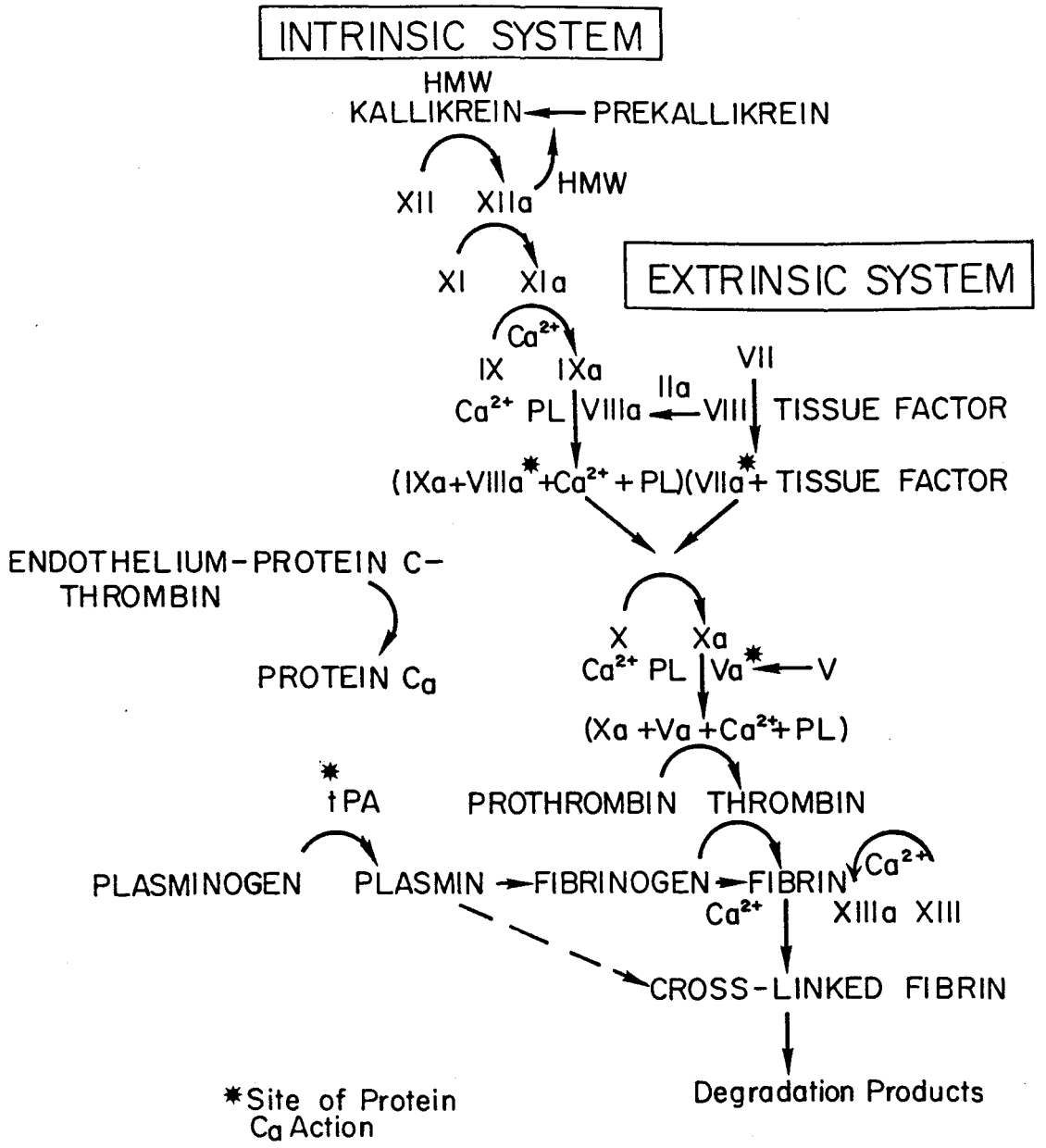
Yurt, R.W., Leid, R.W.S., Austen, K.F., and Silbert, J.E. Native Heparin from Rat Peritoneal Mast Cell. Journal of Biological Chemistry, 253:6687, 1978.

Zar, J.H.: Biostatistical Analysis. Prentice-Hall Inc., New Jersey, 1984.

Ziv, E., Eldor, A., Kleinman, Y., Bar-On, H. and Kidron, M.: Bile Salts Facilitate the Absorption of Heparin From the Intestine. Biochemical Pharmacology, 32:(5) 773-776, 1983.

APPENDIX I

The Coagulation Cascade



APPENDIX II

1, Rue de l'Abbaye - B.P. N° 4 - Notre-Dame de Bondeville
Tél. (16.35) 74.34.00 - Telex 180332 - R.C. Rouen 71 B 50

LABORATOIRE CHOAY

APPENDIX II
Analytical Profile of Heparin (H503)

V/Réf.

N/Réf.

Notre-Dame de Bondeville, le 27 Février 1985

SERVICE ANALYTIQUE

BULLETIN DE CONTROLE

Produit :	Lot : H.503
HEPARINE	Analyse : CF.46.I84 P.79098
	Code : 2I58

CARACTERES	
— Aspect	: conforme
— Pouvoir rotatoire spécifique	: + 52°8
ESSAI	
— Limpidité de la solution à 5 %	: conforme
— pH de la solution à 5 %	: 6,38
— Métaux lourds	: conforme
— Protéines	: conforme
— Perte à la dessication	: 3,97%
— Cendres sulfuriques	: 40,I%
Dosage de l'azote	: 2,2%
Dosage du soufre	: I0,4%
Activité anticoagulante	
— du produit tel quel	: I65 UI/mg
— du produit anhydre	: I7I,8 UI/mg
Essai des pyrogènes	: apyrogène (0°37)
Substances histaminiques	: inf. à 10 mcg/g
Cryoscopie (5 000 UI/ml)	: 0°I24

p.o. X. JAMET
Pharmacien

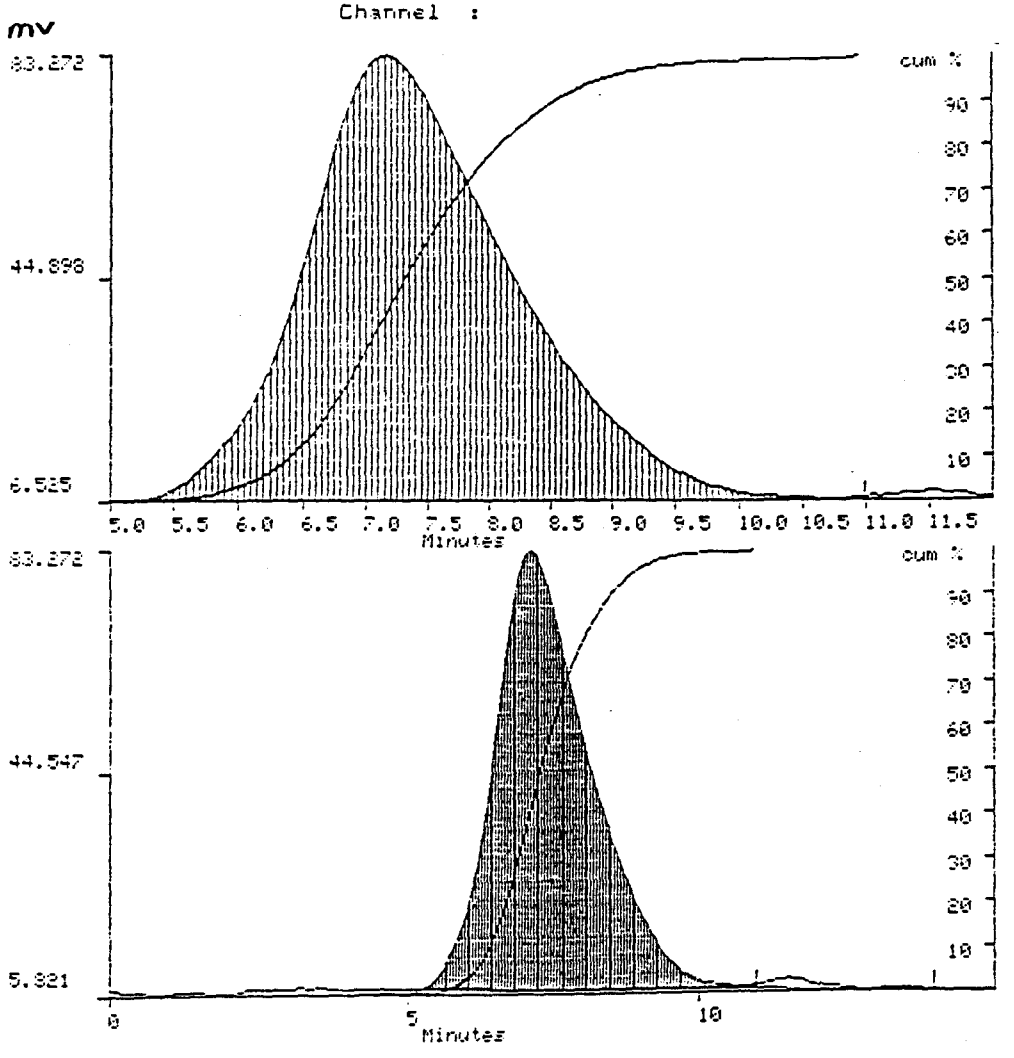
produits
pharmaceutiques

APPENDIX III

APPENDIX III
Molecular Weight Profile of Heparin

Acquisition method	gagt_uvb_	Quantitation method	gagt_uvb_
Units	200ug	System number	1
Channel	1	Vial	9
Injection	1	Total injections	1
Run time	15.00 min	Sample rate	1 per sec
Injection volume	20 uL	Sample amount	
Internal standard amt		Scale factor	
Mode	Analysis	Response factors	Replace
Version	REV 4.0	Channel to calibrate	1
Description			

COLONNE: TSK2000SW VIII SOLVANT : Na2SO4 0.5M
DEBIT : 1ml/mn Channel1 : UV 205nm
Channel :



Molecular Weight Distribution Averages

Weight Average	11392	Number Average	9322
Z Average	17020	Dispersivity	1.222
Viscosity Average	11392	Intrinsic Viscosity	0.000
Z + 1 Average	11e710	Peak Mol Weight	10990

APPENDIX IV

APPENDIX IV

Analytical Profile of Cy 216

46, Avenue Théophile Gautier 75782 Paris - Cedex 16
Tél. 524.46.77

INSTITUT CHOAY

CONTROL SHEET
N° 131813

PRODUCT : CY 216 (D)

REFERENCE : Batch P 533 XH

	Limits	Control
CHARACTERS	White powder, soluble in water	Passes
IDENTIFICATION		
A. Coloured reaction	Positive	Passes
B. Specific optical rotation	Not less than +35°	+49°8
C. Calcium reactions	Positive	Positive
D. Antithrombin activity	Passes	Passes
TESTS		
Clarity of the solution S	Clear or slightly opalescent	Passes
Colour of the solution S	Not more than J5	Passes
pH of the solution S	5.0 to 7.5	6.54
Heavy metals	Not more than 40 ppm	Less than 40 ppm
Loss on drying	Not more than 8 p.100	2.7 p. 100
Sulphated ash	Not more than 35 p.100	32.5 p. 100
Nitrogen	Not more than 2.5 p.100	1.87 p. 100
Proteins	Passes	Passes
UV extinction	Passes	Passes
Calcium	Not less than 7 p.100	9.43 p.100
Fractionation conformity	Conform to the standard	Passes
Nitrites	Not more than 1 ppm	Less than 1 ppm
Boron	Not more than 20 ppm	Less than 20 ppm
Pyrogens	2,000 uAXa IC/kg rabbit	Passes
ASSAY		
Potency anti-factor Xa	Not less than 180uAXa IC per mg calculated with reference to the dried substance	226 uAXa IC/mg

November, 6 1986

C. CURTI
Pharmacist

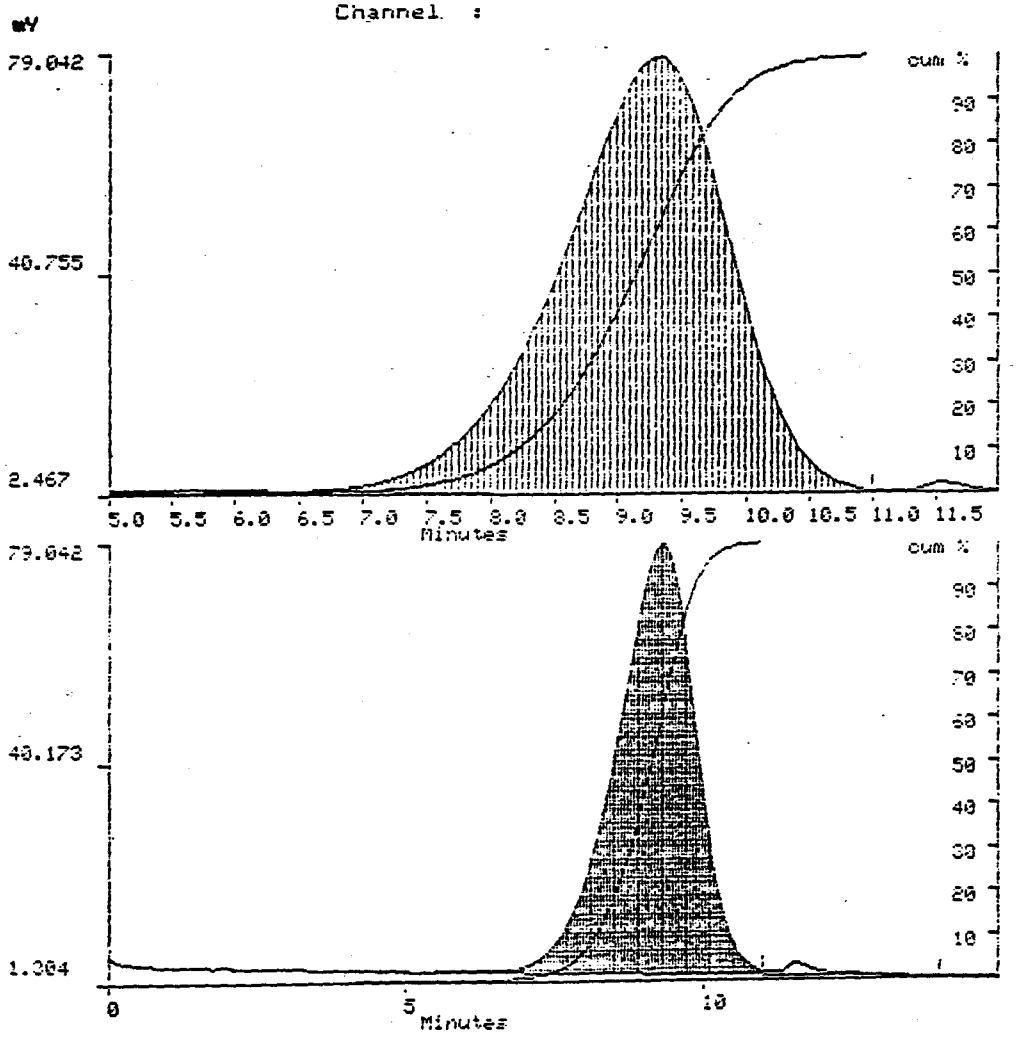
recherche et

APPENDIX V

APPENDIX V
Molecular Weight Profile of CY 216

cquisition method	gagtluv82	Quantitation method	gagtluv82
·.ts	200ug	System number	1
nannel	1	Vial	17
njection	1	Total injections	1
un time	15.00 min	Sample rate	1 per sec
njection volume	20 uL	Sample amount	
nternal standard amt		Scale factor	
ode	Analysis	Response factors	Replace
ersion	REv 4.0	Channel to calibrate	1
escription			

CLONNE: TSK2000SW VIII SOLVANT : Na2SO4 0.5M
EBIT : iml/mn Channel1 : UV 205nm
Channel :



olecular Weight Distribution Averages

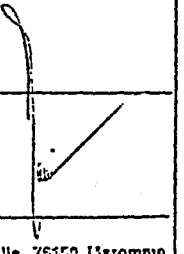
Weight Average	5243	Number Average	4319
Average	16888	Dispersivity	1.214
Viscosity Average	5243	Intrinsic Viscosity	0.000
+ 1 Average	429747	Peak Mol Weight	4586
Mw	3.221	Mz-1 Mw	81.473

APPENDIX VI

BULLETIN DE CONTROLE

Produit : SULFATE DE PROTAMINE suivant projet PE	Lot : 2-85 Analyse : P.78.078 Code : 2198

Caractères	: conformes
Identification :	
A	: conforme
B	: conforme
C	: -81°,3
D	: conforme
E	: conforme
Essais :	
solution S	: limpide,incolore
Absorbance	: conforme
Azote	: 24,7%
Fer	: conforme
Mercure	: conforme
Métaux lourds	: conformes
Sulfates	: 20,35%
Perte à la dessication	: 4,2%
Essai des pyrogènes	: apyrogène (0°,07)
Toxicité anormale	: conforme
Dosage :	
du produit tel quel	: 138 UAH/mg
du produit anhydre	: 144 UAH/mg
Cendres sulfuriques	: 0,2%
Arsenic	: conforme



Conclusion : Conforme
29 Janvier 1985

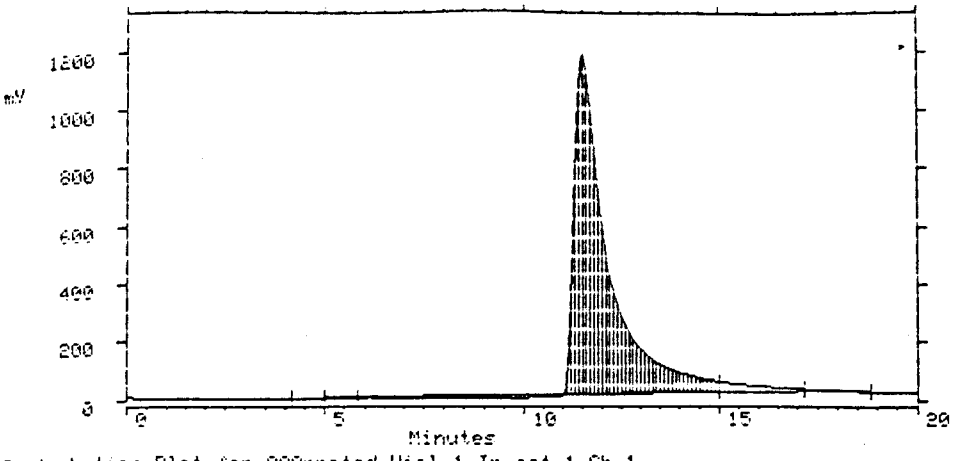
Laboratoire CHOAY, 1, Rue de l'Abbaye - N.-D.-de-Bondeville, 76150 Maromme

1000 MP 0

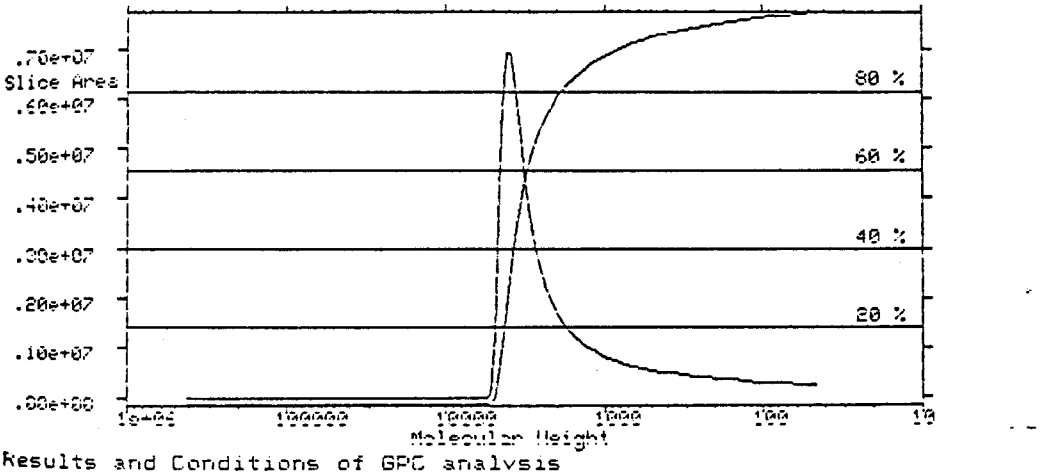
APPENDIX VII

Molecular Weight Profile of Protamine

888protad Vial 1 Inject 1 Ch 1 ,



Distribution Plot for 888protad Vial 1 Inject 1 Ch 1



Results and Conditions of GPC analysis

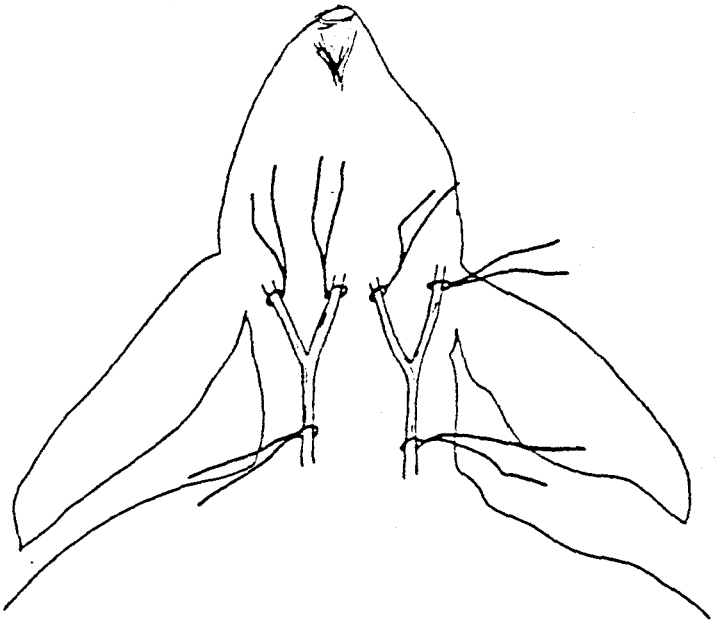
Report Method:	third	Analysis Method:	third
Calibration Method:	third	Normalization:	None
Baseline: Start	5.00 min	End	18.00 min
Number of Data Points in Baseline Region			50
Processing: Start	5.00 min	End	15.00 min
Number of Slices	100	Area Rejection	0
Negative Slices	Disabled		

Molecular Weight calculation by Narrow Standard Calibration Curve.
Mark-Houwink Equation: Not Used.

Number Average	1216	Intrinsic Viscosity	0.0000
Weight Average	3036	Peak Molecular Weight	3953
Z Average	3612	Dispersity	2.496445
Viscosity Average	3036	Z avg / W avg	1.189657
Z + 1 Average	3855	Z+1 avg / W avg	1.269650

APPENDIX VIII

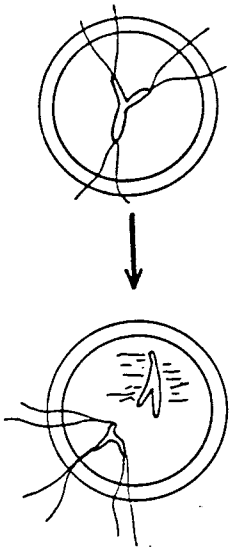
Rabbit Stasis Thrombosis Model Showing Ligatures on Isolated Jugular Veins



(Walenga, J.M., 1987.)

APPENDIX IX

Diagram of the Clot Grading System Used in the Rabbit Stasis Thrombosis Model



0 = no clot all free erythrocytes

± =

1 + =

2+ =

3+ =

4 + =

(Walenga, J.M., 1987.)

APPENDIX X

Rabbit Ear Bleeding Model

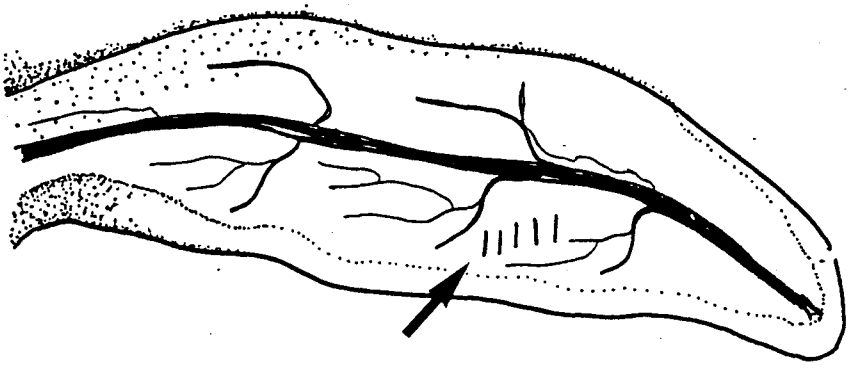


Diagram Showing the Location of Incisions Made in the Rabbit Ear Blood Loss Model.

APPENDIX XI

CUMULATIVE RESULTS OF PRIMATE BLOOD CHEMISTRY PROFILES

Date of Blood Draw	6-7-88	11-7-88	Normal Range*
CL (meq/l)	116.0± 10.4	110.7± 2.5	84.0 - 126.0
K^+ (meq/l)	4.1± 0.7	8.2±13.6	2.3 - 6.7
Na^+ (meq/l)	149.8± 12.8	151.4± 3.2	102.0 - 166.0
Total bilirubin (mg/dl)	0.1± 0.3	0.3± 0.3	0.1 - 2.0
GOT (IU/l)	60.9± 53.3	N/A	16.0 - 97.0
GPT (IU/l)	114.4±236.3	N/A	0.0 - 68.0
Creatinine (mg%)	1.0± 0.7	0.8± 0.3	0.1 - 2.8
Uric acid (mg%)	0.9± 1.7	1.4± 1.3	0.1 - 1.4
Ca^{2+} (mg%)	9.6± 1.4	10.0± 0.6	6.9 - 13.0
Alkaline phosphatase (U)	147.4±267.8	147.9±84.7	9.7 - 89.0
GT (IU/l)	72.9±143.2	78.2±43.0	N/A
Total protein (g/dl)	6.8± 1.0	7.2± 0.3	4.9 - 9.3
Triglyceride (mg/dl)	160.8±394.1	215.5±170.6	N/A
Carbohydrate (mm/l)	22.4± 8.7	N/A	N/A
Albumin (g/dl)	4.5± 1.4	4.6± 0.3	2.8 - 5.2
BUN (mg%)	16.8± 10.4	17.1± 4.1	8.0 - 40.0
Glucose (mg%)	72.5±159.2	87.7±73.0	46.0 - 178.0
Cholesterol (mg%)	173.8±115.6	178.0±28.8	108.0 - 263.0
LDH (IU/l)	583.3±549.4	819.1±187.4	N/A
Phosphorous (mg%)	3.3±3.1	3.2±1.3	3.1 - 7.1

Primate blood chemistry profiles were performed before beginning the pharmacodynamic studies and at their completion by the Loyola University Clinical Chemistry Department. Values represent x ± S.D. N=12 for 6-7-88, N=10 for 11-7-88.

*Normal ranges for rhesus monkeys were obtained from McClure (1975)
N/A - not available.

CUMULATIVE RESULTS OF PRIMATE HEMATOLOGIC PROFILES

	6-7-88	11-7-88	Normal Range*
WBC ($x10^3$)	5.8±1.4	6.1±1.3	2.5 - 26.7
RBC ($x10^6$)	5.6±0.7	5.4±0.7	3.6 - 7.0
HGB (g/dl)	13.5±1.4	12.4±1.9	8.8 - 16.5
HCT (%)	40.4±3.5	37.8±4.8	26.0 - 48.0
MCV (μm^3)	72.0±4.2	70.0±6.7	58.1 - 116.9
MCH ($\mu\mu g$)	24.0±1.4	22.9±2.6	18.5 - 36.6
MCHC (g/dl)	33.4±2.8	32.6±1.3	25.6 - 40.2
RDW	13.8±1.4	14.1±2.6	N/A
PLT ($x10^3$)	357.7±84.4	173.3±168.9	109.0 - 597.0
MPV (μm^3)	9.3±2.7	10.0± 1.0	N/A
Lymphocyte (%)	20.5±106.2	14.1± 9.0	8.0 - 92.0
Lymphocyte ($x10^3$)	1.2±6.6	2.8± 1.0	0.69 - 14.5
TPA (ng/ml)	N/A	1.5±0.6	N/A

Primate blood hematologic profiles were performed before beginning the pharmacodynamic studies and at their completion by the Loyola University Clinical Chemistry Department. Values represent x ± S.D. N=12 for 6-7-88, N=10 for 11-7-88.
*Normal ranges for rhesus monkeys were obtained from McClure (1975).
N/A - not available

APPENDIX XIc

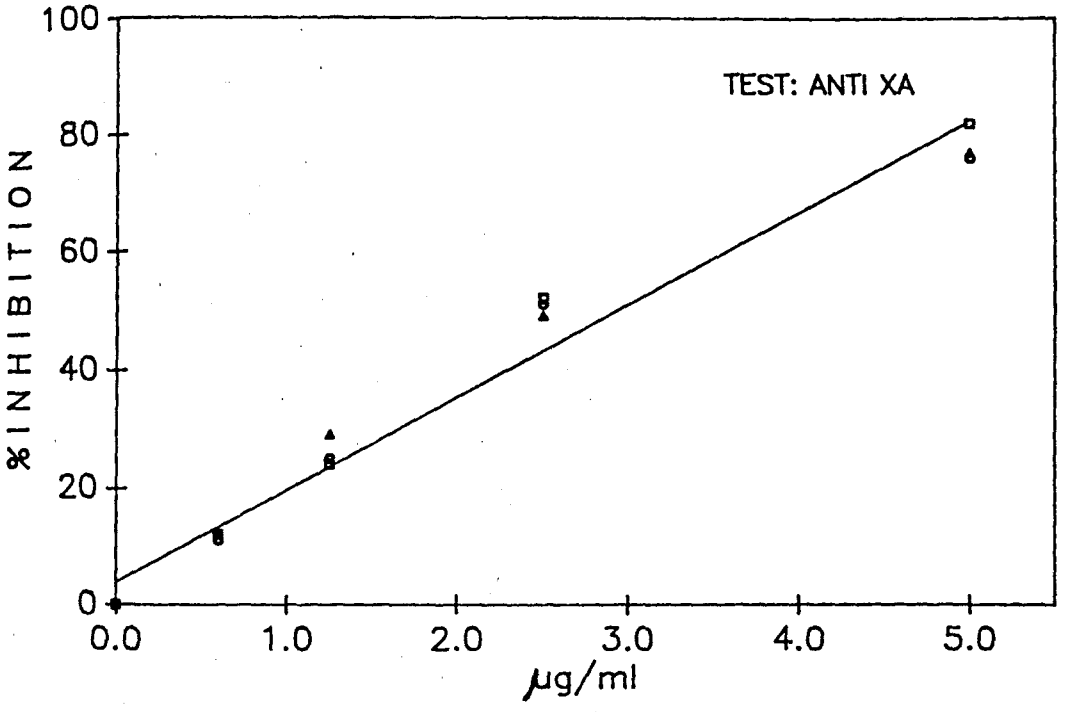
CUMULATIVE RESULTS OF PRIMATE COAGULATION PROFILES

	6-7-88	11-7-88
APTT (seconds)	27.4±4.2	29.6±2.6
TT (5U/ml) (seconds)	30.9±5.2	24.8±4.8
Heptest (seconds)	21.5±3.1	22.5±1.3

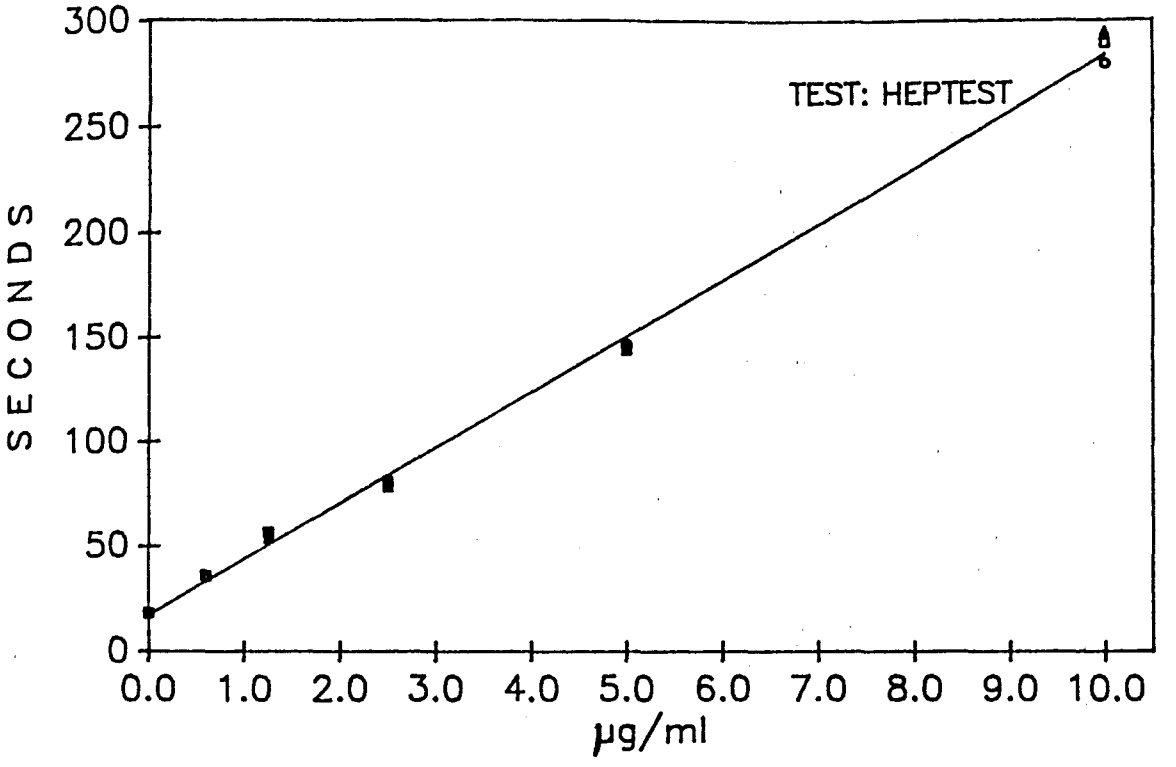
Primate coagulation profiles were performed before beginning the pharmacodynamic studies and at their completion. Values represent x ± S.D. N=12 for 6-7-88, N=10 for 11-7-88.

APPENDIX XII

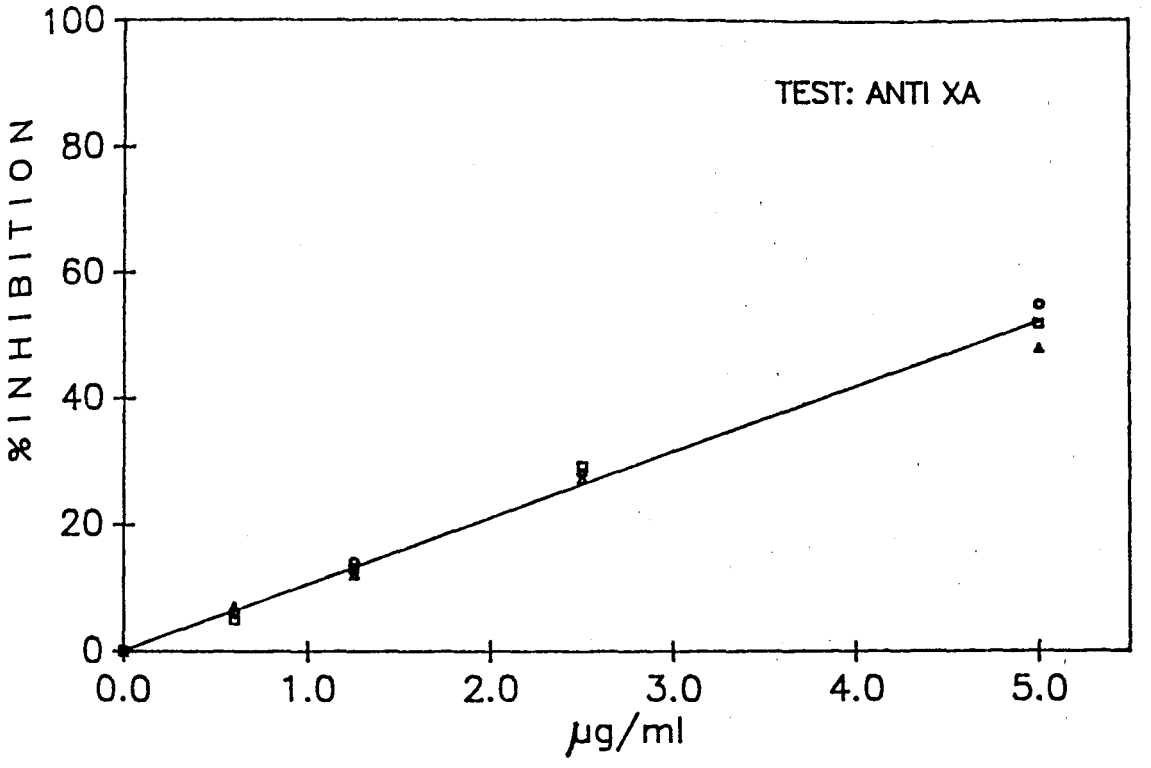
CALIBRATION CURVE OF HEPARIN SUPPLEMENTED TO NMP
USED TO CALCULATE PHARMACODYNAMIC TIME COURSE PARAMETERS



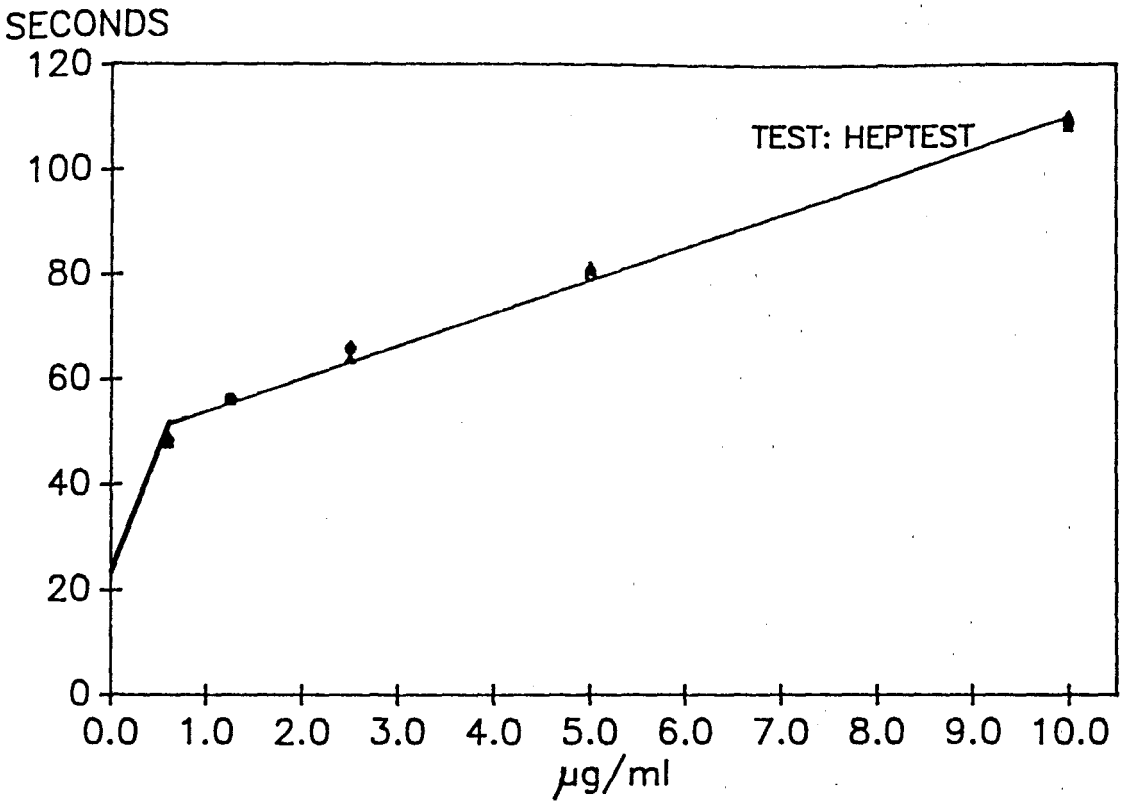
CALIBRATION CURVE OF HEPARIN SUPPLEMENTED TO NMP
SED TO CALCULATE PHARMACODYNAMIC TIME COURSE PARAMETERS

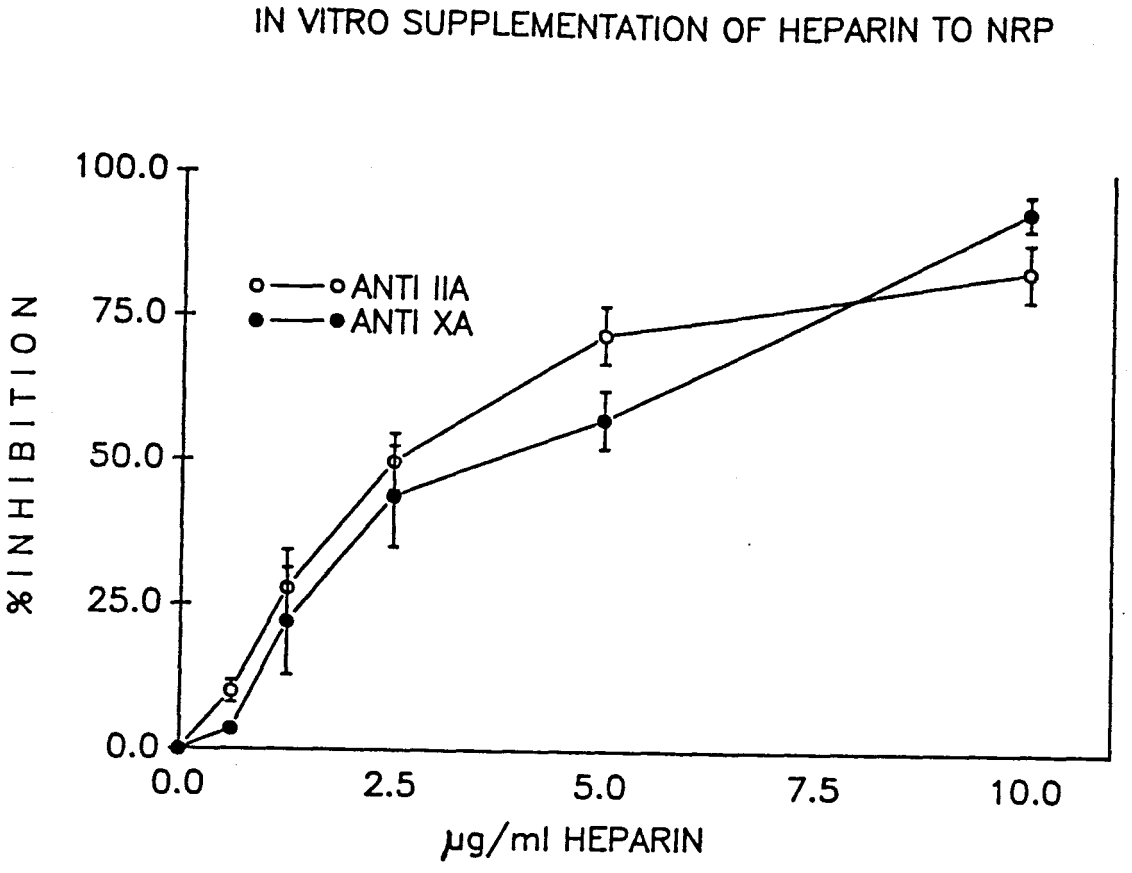


CALIBRATION CURVE OF CY 216 SUPPLEMENTED TO NMP
USED TO CALCULATE PHARMACODYNAMIC TIME COURSE PARAMETERS

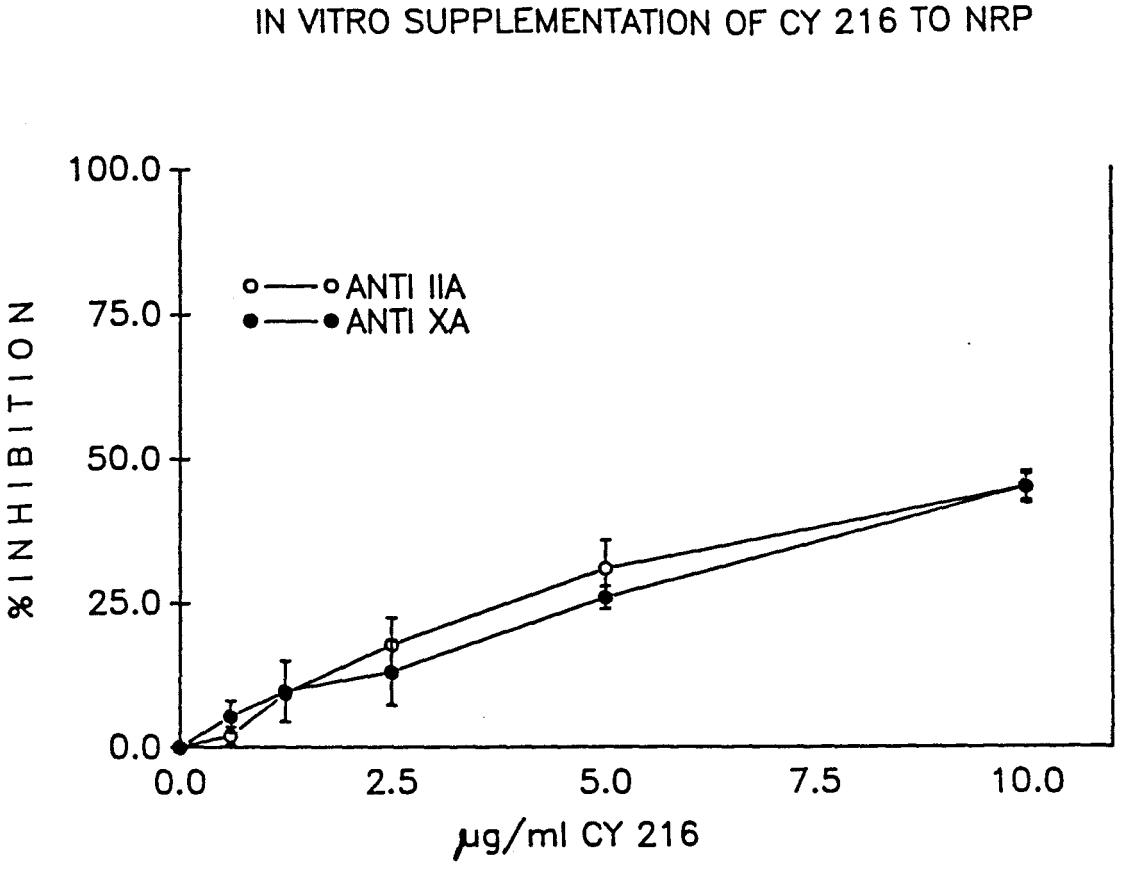


CALIBRATION CURVE OF CY 216 SUPPLEMENTED TO NMP USED TO CALCULATE PHARMACODYNAMIC PARAMETERS





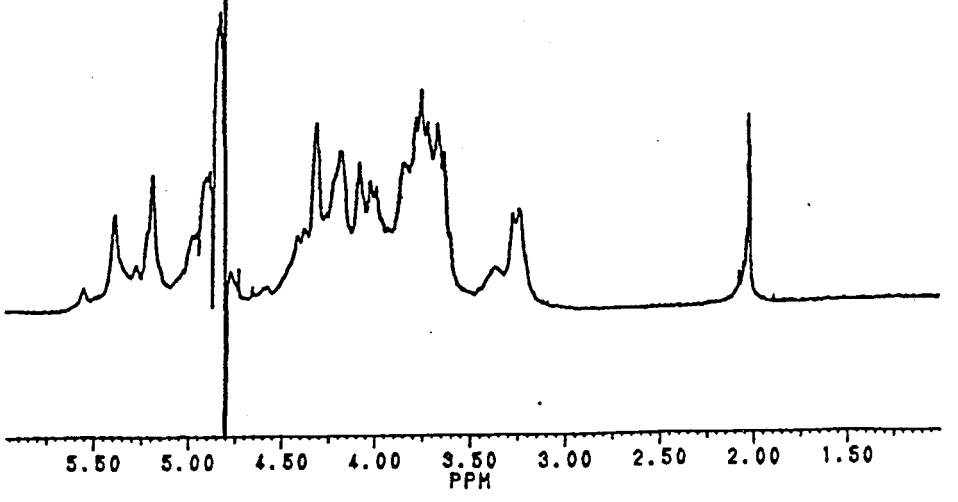
Heparin was supplemented to NRP in a concentration range of 0-10 µg/ml. Antiprotease activity was determined by the amidolytic anti Xa and anti IIa methods. Data represents a mean ± S.E. The experiment was performed in triplicate on separate days.



CY 216 was supplemented to NRP in a concentration range of 0-10 µg/ml. Antiprotease activity was determined by the amidolytic anti Xa and anti IIa methods. Data represents a mean ± S.E. The experiment was performed in triplicate on separate days.

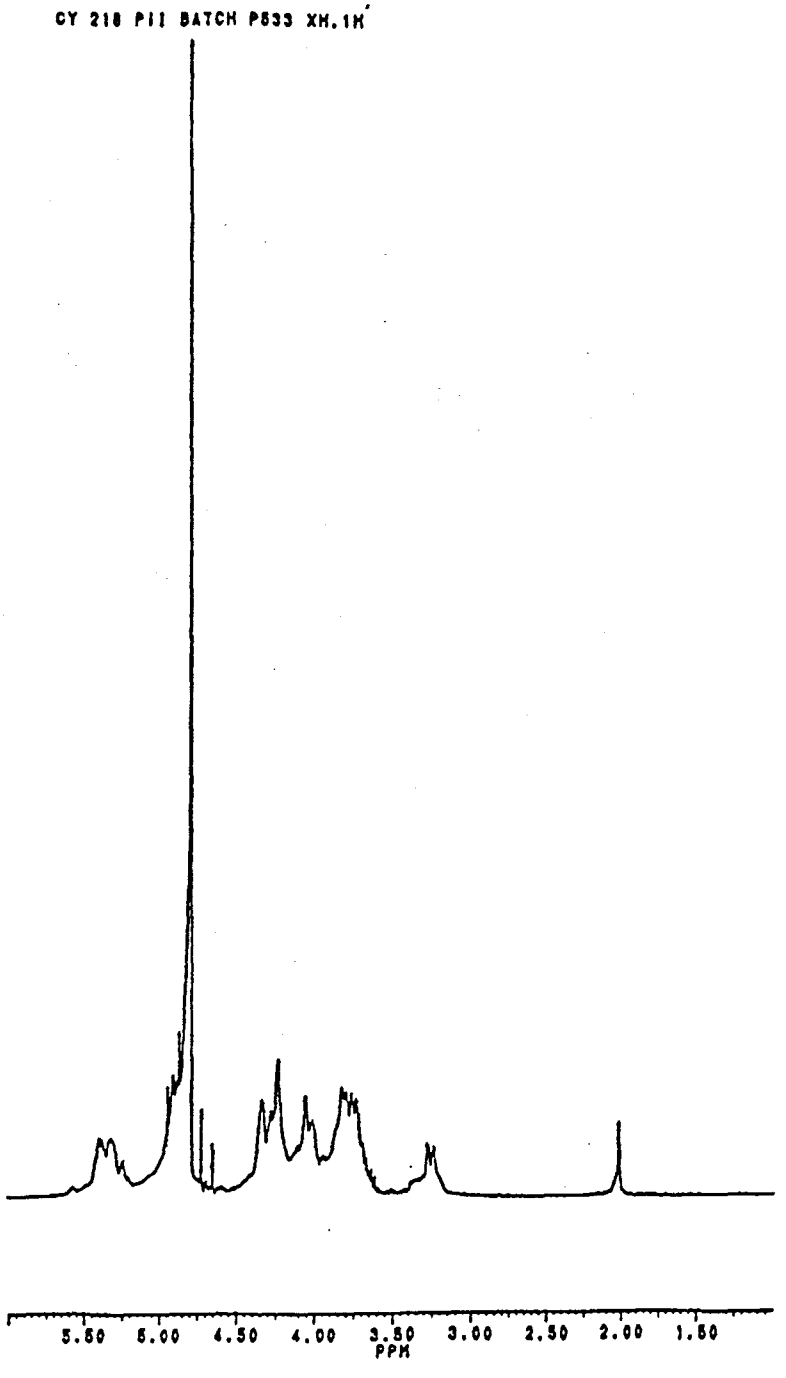
APPENDIX XIII

Heparin H 503
Proton NMR (300 MHz) in D_2O



^1H NMR Spectrum (300 MHz) in D_2O

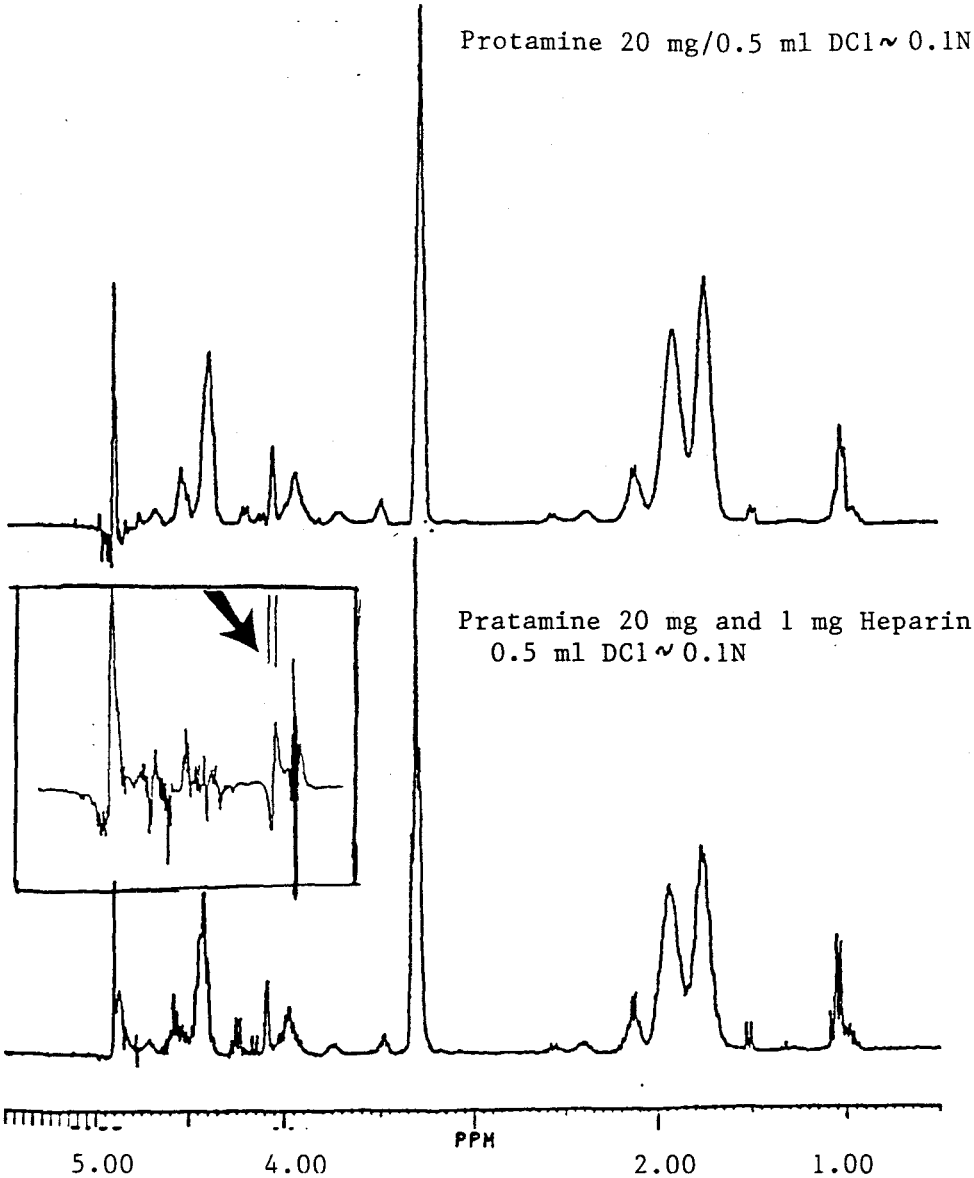
APPENDIX XIV



^{1}H NMR Spectrum (300 MHz) in D_2O

APPENDIX XV

^{1}H NMR SPECTRA (300 MHz) in DCl



^{1}H NMR Spectra (300 MHz) in DCl. The upper spectrum is protamine. The lower spectra is heparin and protamine. The box is an enlargement of the lower spectrum in the range of 3-5 ppm. The arrow represents an area of change in the chemical shift as compared to the upper spectrum.

APPENDIX XVI

CONVERSION OF GRAVIMETRIC AMOUNTS OF HEPARIN, CY 216 AND PROTAMINE TO APPROXIMATE MOLAR RANGES

Species	Conc. (μgml) Dose (mg/kg)	Heparin (μg/moles)	CY 216 (μg/moles)	Protamine (μg/moles)
In Vitro	0.6	.01-0.32	.04-0.86	.09-0.40
	1.25	.02-0.66	.07-1.80	.18-0.83
	2.50	.04-1.31	.15-3.60	.36-1.67
	5.0	.09-2.63	.30-7.20	.72-3.33
	10.0	.18-5.26	.59-14.3	1.4-6.66
Primate	0.7	.01-.37	.04-1.00	.10-.47
	1.4	-	-	.20-.93
	2.1	-	-	.30-1.40
Rabbit	0.1	.002-.05	.006-.14	.01-.07
	0.2	.004-.11	.01-.29	.03-.13
	1.0	.02-.53	.06-1.4	.14-.67
	2.0	.04-1.05	.12-2.9	.29-1.33
	2.5	.04-1.32	.15-3.6	.36-1.7
Rat	.32	.010-.168	.02-.46	.05-.21
	.64	.012-.337	.04-.91	.09-.43
	.72	.013-.379	.042-1.03	.10-.48
	1.0	.018-.526	.06-1.43	.14-.67
	2.0	.036-1.052	.12-2.86	.29-1.3

The following molecular weight ranges were used for the ocnversion from gravimeter to molar amounts. Heparin (57,000 - 1,900) daltons; CY 216 (17,000 - 700) daltons; Protamine (7,000 - 1,500) daltons.

APPENDIX XVII

APPENDIX XVII

DOSAGES OF HEPARIN, CY 216 AND PROTAMINE WHICH WERE ADMINISTERED DURING THE ANIMAL EXPERIMENTS AS COMPARED TO DOSAGES USED DURING CARDIOPULMONARY BYPASS

Species	Drug	Weight(kg)	Dose (mg/kg)	Total mg	Blood Volume (mg/kg b.w.)	Drug Concentration (μg/ml)
Primate	heparins	8.0	0.70	5.6	75[c]	9
Rabbit	heparins	2.5	0.10	0.25	53[c]	2
			2.0	5.0		38
Rat	heparins	0.25	1.0	0.25	65[c]	15
			2.5	0.63		39
Man	Heparin	70	2.0[a]-3.0[b]	140.0	78.6[d]	25-38
	Protamine		2.5[a]	175.0		32

Concentrations were calculated for dosages of heparin, CY 216 and protamine which were used during the experiments and which are used for man during cardiopulmonary bypass procedures.

a. Dosages used at Foster McGaw Hospital.
b. Physician's Desk Reference 1989.
c. Davis P. Personal Communication
d. Goodman and Gilman's The Pharmacological Basis of Therapeutics. MacMillan Publishing Company, 1985.

APPENDIX XVIII

RATIO OF PROTAMINE TO HEPARIN AT WHICH COMPLETE NEUTRALIZATION OCCURRED

Test	In Vitro (NHP)	In Rabbits	In Primates
APTT	1:1	1:1	1:1
Thrombin Time	1:1	3:2	1:1
Heptest^{R}	2:1	3:2	1:1
Anti-Xa	2:1	1:1	1:1
Anti-IIa	2:1	1:1	1:1

Ratios were calculated from data obtained from the rabbit ear blood loss experiments and the primate pharmacodynamic time course experiments. Blood was drawn 5 minutes after the administration of protamine in the rabbit experiment and 15 minutes after the administration of protamine in the primate experiments.

APPENDIX XIX

APPENDIX XIX

RATIO OF PROTAMINE TO CY 216 AT WHICH COMPLETE NEUTRALIZATION OCCURRED

Test	In Vitro NHP	In Rabbits	In Primates
APTT	2:1	1:1	1:1
Thrombin Time	1:2	3:2	1:1
Heptest^{R}	>2:1	>3:2	1:1
Anti-Xa	>2:1	>2:1	2:1
Anti-IIa	2:1	1:1	1:1

Ratios were calculated from data obtained from the rabbit ear blood loss experiments and the primate pharmacodynamic time course experiments. Blood was drawn 5 minutes after the administration of protamine in the rabbit experiment and 15 minutes after the administration of protamine in the primate experiments.

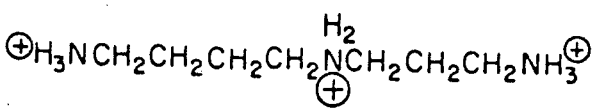
APPENDIX XX

The Amino-Acid Sequences of Components of Salmine

Table VIII-6. Peptides obtained by Neutral Protease digestion
Salmine A1

Structure	Recovery (mole/mole)
Pro·Arg·Arg·Arg·Arg Pro·Arg·Arg·Arg·Arg·Ser	0.37
Arg·Ser	0.42
Pro·Arg·Arg·Arg	0.44
(Ser)·Ser·Ser·Arg·Pro	0.96
Val·Arg·Arg·Arg·Arg·Arg·Pro·Arg	0.80
Arg·Arg·Pro·Arg	0.12
Val·Arg·Arg·Arg	0.07
Val·Ser·Arg·Arg·Arg·Arg·Arg·Arg Val·Ser·Arg·Arg·Arg·Arg·Arg·Arg·Gly	0.83
Val·Ser·Arg·Arg·Arg	0.10
Gly·Gly·Arg·Arg·Arg·Arg	0.48
Gly·Arg·Arg·Arg·Arg	0.50

(Adapted from Ando T. et. al. Protamine - Isolation. Characterization, Structure and Function, Springer, Berlin Heidelberg, New York, 1973).



SPERMIDINE

(Dentsman S.C. et. al. Biochemical and Biophysical Research Communication 149:(1) 194-201, 1987).

APPENDIX XXI

Noncompartmental Model Diagram

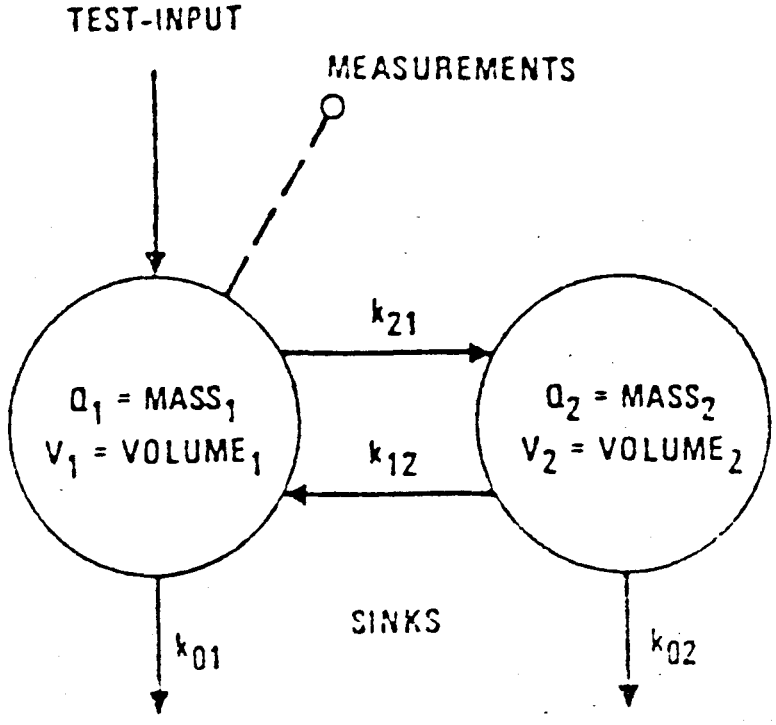
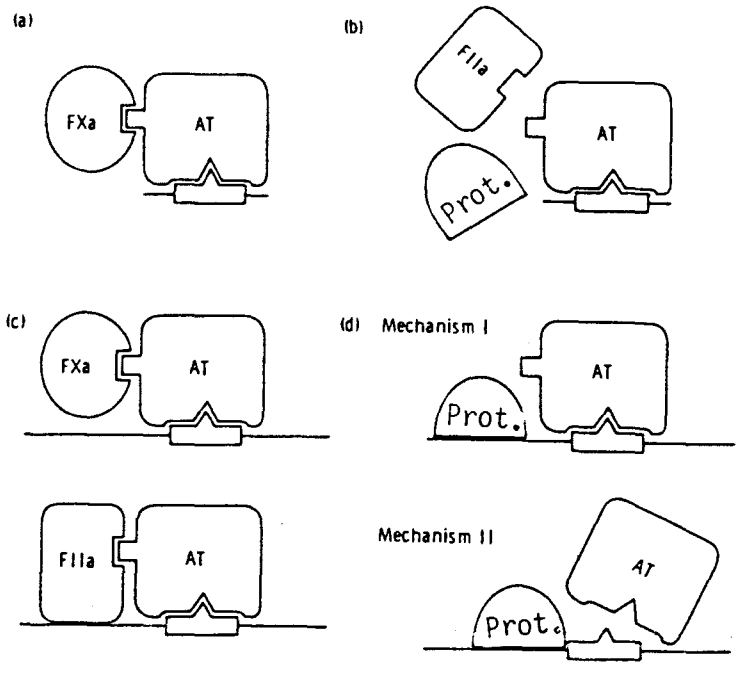


FIG. 2. Most general 2-compartment (2-pool) model, with input and output in the same pool.

(Bourne D.W.A. et.al. Pharmacokinetics for the non-mathematical, MTP Press Limited, Lancaster, Australia, 1988).

APPENDIX XXII

Interactions of an Oligosaccharide Factor Xa, Thrombin, AT III and Protamine



Possible interactions of an oligosaccharide, the approximate size of the antithrombin binding region, and of a full-sized heparin chain, with antithrombin (AT), factor Xa (Xa), thrombin (IIa) and protamine (prot.). a) an oligosaccharide containing only the antithrombin binding sequence binds to AT and accelerates the inhibition of Xa but not IIa (b). It cannot bind to protamine and its anti-factor Xa activity is therefore resistant to neutralization. (c). Protamine can bind to a full-sized heparin and neutralize its anti-factor Xa and antithrombin activities. Two mechanisms (d) can be proposed to explain this neutralizing action. In mechanism I the neutralizing protein binds to a site adjacent to the antithrombin binding site and sterically hinders formation of the antithrombin-proteinase complex. Alternatively, in mechanism II the neutralizing protein binds immediately adjacent to (and perhaps overlapping with) the antithrombin binding site and displaces the inhibitor from the polysaccharide chain. (Adapted from Lane D.A., Heparin Neutralizing Proteins in Blood. Structure and Activities of Heparin and Related Mucopolysaccharides. Annals of the New York Academy of Sciences 566: 473-475, 1989.)

The differential neutralization of Fraxiparine^{R} observed in the Xa (Heptest and amidolytic Xa) assays may be due to the fact that oligosaccharides (mw <5,000) comprise almost 80% of this agent. Thus, protamine is unable to completely neutralize these activities effectively and residual activity is observed. On the other hand, the anti-IIa activities as measured by thrombin time and AIIa are effectively neutralized by protamine.

APPENDIX XXIII

APPENDIX XXIIIa

IN VITRO SUPPLEMENTATION OF HEPARIN TO NHP

Concentration (μg/ml)	APTT (seconds)	Heptest (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
10.0	>150	228.5 ± 14.6	90.8 ± 0.6	91.0 ± 1.0
5.0	>150	116.0 ± 2.2	72.0 ± 3.0	84.2 ± 2.4
2.5	80.4 ± 4.4	78.3 ± 1.2	49.8 ± 4.7	58.8 ± 2.9
1.25	50.9 ± 4.1	56.3 ± 1.7	38.8 ± 5.7	44.8 ± 7.5
0.6	37.5 ± 1.3	32.7 ± 2.7	16.7 ± 4.4	15.8 ± 3.7
0.0	30.5 ± 2.2	15.5 ± 1.2	0 ± 0	0 ± 0

Data represents a mean ± S.E., N=5.

APPENDIX XXIIIb

IN VITRO SUPPLEMENTATION OF HEPARIN TO NMP

Concentration (μg/ml)	APTT (seconds)	Heptest (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
10.0	>150	287.9 ± 7.6	94.3 ± 4.0	92.7 ± 1.6
5.0	>150	145.3 ± 1.6	80.3 ± 3.2	88.0 ± 1.7
2.5	78.5 ± 1.3	78.9 ± 1.3	50.3 ± 1.2	83.3 ± 3.5
1.25	42.6 ± 1.2	54.0 ± 1.5	26.0 ± 2.6	50.3 ± 2.5
0.6	32.7 ± 1.2	35.5 ± 0.5	11.7 ± 0.6	35.7 ± 2.5
0.0	24.5 ± 1.6	18.0 ± 0.3	0 ± 0	0 ± 0

Data represents a mean ± S.E., N=3.

APPENDIX XXIIIc

IN VITRO SUPPLEMENTATION OF HEPARIN TO NRP

Concentration (μg/ml)	APTT (seconds)	Heptest (seconds)	Anti-Xa (% inhibition)	Anti-IIa (%inhibition)
10.0	>300	>300	93.3 ± 3.0	83.0 ± 5.0
5.0	>279.3 ± 21.1	>300	57.3 ± 5.1	72.0 ± 9.0
2.5	157.7 ± 16.4	188.9 ± 8.3	43.7 ± 8.8	49.7 ± 8.6
1.25	100.4 ± 17.2	60.9 ± 0.6	22.0 ± 9.2	27.7 ± 6.6
0.6	77.7 ± 1.4	40.0 ± 3.3	3.5 ± 0.4	10.0 ± 1.5
0.0	56.6 ± 0.8	20.5 ± 1.7	0 ± 0	0 ± 0

Data represents a mean ± S.E., N=3.

APPENDIX XXIIId

IN VITRO SUPPLEMENTATION OF HEPARIN TO NRatP

Concentration (μg/ml)	APTT (seconds)	Heptest (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
10.0	>150	>300	85.0 ± 0.6	86.3 ± 2.4
5.0	78.1 ± 5.3	163.8 ± 30.9	60.0 ± 3.6	59.3 ± 0.6
2.5	36.4 ± 1.8	100.2 ± 12.5	32.7 ± 3.5	46.0 ± 6.6
1.25	26.1 ± 0.8	76.1 ± 3.3	19.3 ± 4.3	17.7 ± 14.9
0.6	22.6 ± 0.7	69.6 ± 1.5	3.0 ± 7.3	9.3 ± 9.5
0.0	16.1 ± 0.9	56.6 ± 0.8	0 ± 0	0 ± 0

Data represents a mean ± S.E., N=3.

APPENDIX XXIIIe

IN VITRO SUPPLEMENTATION OF CY 216 TO NHP

Concentration (μg/ml)	APTT (seconds)	Heptest (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
10.0	67.2 ± 0.8	112.7 ± 3.2	65.2 ± 3.8	56.2 ± 7.9
5.0	51.2 ± 1.9	81.5 ± 5.5	45.4 ± 4.8	37.0 ± 2.2
2.5	40.8 ± 0.6	66.4 ± 1.2	37.3 ± 2.2	13.6 ± 3.0
1.25	36.7 ± 0.3	54.7 ± 1.6	27.0 ± 3.8	8.4 ± 2.2
0.6	34.8 ± 0.6	38.2 ± 1.6	14.2 ± 6.6	3.4 ± 1.8
0.0	30.5 ± 2.2	15.5 ± 1.2	0 ± 0	0 ± 0

Data represents a mean ± S.E., N=5.

APPENDIX XXIIIf

IN VITRO SUPPLEMENTATION OF CY 216 TO NMP

Concentration (μg/ml)	APTT (seconds)	Heptest (seconds)	Anti- Xa (% inhibition)	Anti-IIa (%inhibition)
10.0	72.7 ± 4.3	>150	71.3 ± 3.1	70.0 ± 6.2
5.0	46.9 ± 0.6	41.0 ± 0	51.0 ± 2.6	53.3 ± 3.2
2.5	35.4 ± 0.8	21.3 ± 0.7	25.6 ± 1.2	34.3 ± 5.1
1.25	32.5 ± 3.3	17.3 ± 0.1	12.3 ± 0.6	22.3 ± 4.6
0.6	27.2 ± 1.2	15.0 ± 0.0	6.0 ± 1.0	16.0 ± 2.6
0.0	24.5 ± 1.6	18.0 ± 0.3	0 ± 0	0 ± 0

Data represents a mean ± S.E., N=3.

APPENDIX XXIIIg

IN VITRO SUPPLEMENTATION OF CY 216 TO NRP

Concentration (μg/ml)	APTT (seconds)	Heptest (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
10.0	99.9 ± 0	147.5 ± 17.6	31.0 ± 7.1	45.0 ± 2.8
5.0	83.9 ± 5.4	64.5 ± 2.0	26.0 ± 2.0	31.0 ± 5.0
2.5	64.0 ± 1.7	46.5 ± 1.8	13.3 ± 5.7	17.7 ± 4.8
1.25	67.1 ± 3.1	39.1 ± 1.9	9.7 ± 5.3	9.3 ± 0.7
0.6	62.5 ± 3.7	34.6 ± 2.6	5.3 ± 2.8	2 ± 1.6
0.0	56.6 ± 0.8	20.5 ± 1.7	0 ± 0	0 + 0

Data represents a mean ± S.E., N=3.

APPENDIX XXIIIh

IN VITRO SUPPLEMENTATION OF CY 216 TO NRatP

Concentration (μg/ml)	APTT (seconds)	Heptest (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
10.0	43.6 ± 1.9	195.1 ± 2.2	62.7 ± 2.2	60.7 ± 2.8
5.0	31.3 ± 0.9	107.8 ± 3.2	38.0 ± 1.6	38.0 ± 3.6
2.5	25.1 ± 0.1	82.8 ± 2.2	20.3 ± 2.1	25.7 ± 3.0
1.25	22.8 ± 0.3	75.3 ± 1.1	8.7 ± 4.6	17.7 ± 12.4
0.6	21.7 ± 0.1	72.9 ± 1.2	6.3 ± 1.5	5.3 ± 5.4
0.0	16.1 ± 0.9	56.6 ± 0.8	0 ± 0	0 ± 0

Data represents a mean ± S.E., N=3.

APPENDIX XXIIIi

IN VITRO ANTICOAGULANT AND ANTIPROTEASE ACTIVITY OF HEPARIN AFTER PROTAMINE ADMINISTRATION

Plasma system: NHP
Heparin Concentration: 5 μg/ml

Protamine Concentration (μg/ml)	APTT (seconds)	Thrombin Time (5U/ml) (seconds)	Heptest (seconds)	Anti-Xa % Inhibition	Anti-IIa % Inhibition
10.0	40.6 ± 1.1	27.8 ± 3.2	30.7 ± 3.5	1.2 ± 1.1	2.5 ± 1.5
5.0	43.6 ± 1.7	37.8 ± 5.4	45.7 ± 7.8	7.8 ± 2.7	12.8 ± 4.5
2.5	94.9 ± 7.3	>150	86.7 ± 3.6	40.6 ± 5.1	42.6 ± 7.3
0	>150	>150	119.0 ± 9.6	67.2 ± 1.6	79.0 ± 0.0
Saline control	33.2 ± 0.4	27.2 ± 0.7	17.6 ± 1.6	0 ± 0	0 ± 0

Data represents a mean ± S.E., N=5.

APPENDIX XXIIIj

IN VITRO ANTICOAGULANT AND ANTIPROTEASE ACTIVITY OF CY 216 AFTER PROTAMINE ADMINISTRATION

Plasma system: NHP
CY 216 Concentration: 5 μg/ml

Protamine Concentration (μg/ml)	APTT (seconds)	Thrombin Time (5U/ml) (seconds)	Heptest (seconds)	Anti-Xa % Inhibition	Anti-IIa % Inhibition
10.0	46.6 ± 1.4	33.3 ± 2.6	69.0 ± 1.5	25.2 ± 4.9	4.8 ± 3.6
5.0	49.2 ± 1.5	37.8 ± 4.0	77.0 ± 3.7	28.3 ± 1.0	5.8 ± 2.5
2.5	49.8 ± 1.3	47.0 ± 3.3	76.4 ± 4.6	30.2 ± 1.8	17.0 ± 3.6
0	55.5 ± 1.4	>150	87.5 ± 2.5	38.5 ± 2.6	30.8 ± 3.2
Saline control	33.2 ± 0.4	27.2 ± 0.7	17.6 ± 1.6	0 ± 0	0 ± 0

Data represents a mean ± S.E., N=5.

APPENDIX XXIV

APPENDIX XXIVa

CUMULATIVE RESULTS OBTAINED IN A LASER-INDUCED THROMBOSIS MODEL

Treatment	N	$\bar{x}$ + S.E.
Control (power = 1.8, objective = 1/30)	11	2.3 ± 0.04
Control (power = 1.2, objective = 1/15)	5	3.1 ± 0.2
Heparin 1 mg/kg i.v.	4	5.1 ± 0.23
Heparin 1 mg/kg i.v. and Protamine 0.32 mg/kg i.v.	6	5.7 ± 0.22
Heparin 1 mg/kg i.v. and Protamine 0.64 mg/kg i.v.	5	3.2 ± 0.16
CY 216 1 mg/kg i.v.	5	3.9 ± 0.08
CY 216 1 mg/kg i.v. and Protamine 0.74 mg/kg i.v.	7	2.5 ± 0.20
Protamine 0.74 mg/kg i.v.	3	4.8 ± 0.45
Heparin 10 mg/kg i.v.	3	from 5 to >15
Heparin 10 mg/kg s.c.	5	5.8 ± 0.12
CY 216 10 mg/kg s.c.	5	5.2 ± 0.26

CUMULATIVE BLEEDING TIMES OBTAINED IN A RAT TAIL BLEEDING MODEL

Treatment	N	Tail Transection Time (seconds)
Saline	9	355.0 ± 27.7
Protamine 1 mg/kg i.v.	7	531.4 ± 48.3
Heparin 2 mg/kg i.v.	7	677.1 ± 87.1
CY 216 2 mg/kg i.v.	7	272.1 ± 28.2
Heparin 2 mg/kg i.v. and protamine 2 mg/kg i.v.	7	406.4 ± 46.2
CY 216 2 mg/kg i.v. and protamine 2 mg/kg i.v.	7	340.7 ± 50.7
Heparin 2.5 mg/kg s.c.	7	324.3 ± 51.7
CY 216 2.5 mg/kg s.c.	7	255.0 ± 40.2
Heparin 2.5 mg/kg s.c. and protamine 2.5 mg.kg i.v.	7	405.0 ± 54.2
CY 216 2.5 mg/kg s.c. and protamine 2.5 mg/kg i.v.	7	356.4 ± 64.3

Data represents a mean ± S.E.

APPENDIX XXIVc

EX VIVO ACTIVITY AND BLOOD LOSS VALUES AFTER PROTAMINE ADMINISTRATION TO RABBITS

Time: Pre-challenge

Treatment	N	APTT (seconds)	TT 5U (seconds)	Heptest (seconds)	Anti-Xa (% Inhibition)	Anti-IIa % Inhibition	Blood Loss (RBCs x 10^9/ liter)
Control	5	83.2±11.5	30.1±3.3	24.8±3.0	0	0	0.10±0.01
Protamine 3 mg/kg i.v.	4	133.9± 5.4	37.0±1.5	26.4±3.7	2.0±1.7	0	0.2±0.1
Protamine 2 mg/kg i.v.	3	115.4±12.9	33.2±7.3	22.4±0.7	0	0	0.04±0.01

Data represents a mean ± S.E.

APPENDIX XXIVd

EX VIVO ACTIVITY AND BLOOD LOSS VALUES AFTER HEPARIN AND PROTAMINE ADMINISTRATION TO RABBITS

Treatment	N	APTT (seconds)	TT 5U (seconds)	Heptest (seconds)	Anti-Xa (% Inhibition)	Anti-IIa (% Inhibition)	Blood Loss (RBCs x 10^9/ liter)
Heparin 1 mg/kg i.v.	6	>297.9±2.0	>300	>271.5±5.0	23.8±10.4	64.8±15.3	0.48±0.15
Heparin 2 mg/kg i.v.	14	>300	>300	>300	70.2± 4.9	92.4± 0.8	0.80±0.11
Heparin 2 mg/kg i.v. with Protamine 2 mg/kg i.v.	6	82.7±11.5	160.5±38.9	166.0±55.5	3.0±3.0	5.8± 3.9	0.36±0.11
Heparin 2 mg/kg i.v. with Protamine 3 mg/kg i.v.	5	87.6±7.5	33.4±2.4	58.4± 3.7	0.0±0.0	0.8±0.8	0.09±0.02

Data represents a mean ± S.E.

APPENDIX XXIVe

EX VIVO ACTIVITY AND BLOOD LOSS VALUE AFTER CY 216 AND PROTAMINE ADMINISTRATION TO RABBITS

Treatment	N	APTT (seconds)	TT 5U (seconds)	Heptest (seconds)	Anti-Xa (% Inhibition)	Anti-IIa (% Inhibition)	Blood Loss (RBCs x 10^9 liter)
CY 216 1 mg/kg i.v.	5	>150	>150	138.1±7.0	42.3±2.0	49.5±1.8	0.42±0.10
CY 216 2 mg/kg i.v.	14	>150	>150	>296.1±3.9	50.9±6.9	72.1±3.0	0.30±0.08
CY 216 3 mg/kg i.v.	5	>150	>150	>150	65.0±6.9	82.8±5.8	0.84±0.22
CY 216 2 mg/kg i.v. with Protamine 2 mg/kg i.v.	5	111.3±24.5	118.6±47.5	138.1±45.5	26.0±14.0	25.8±7.8	0.06±0.02
CY 216 2 mg/kg i.v. with Protamine 3 mg/kg i.v.	5	108.1±14.5	47.1±10.5	153.0±47.1	12.6±4.8	0.14±0.17	0.06±0.02

Data represents a mean ± S.E.

APPENDIX XXIVf

EX VIVO ACTIVITY AND BLOOD LOSS VALUES AFTER HEPARIN OR CY 216 AND PROTAMINE ADMINISTRATION TO RABBITS

Treatment	N	APTT (seconds)	TT 5U (seconds)	Heptest (seconds)	Anti-Xa (% Inhibition)	Anti-IIa (% Inhibition)	Blood Loss (RBCs x 10^9 liter)
Heparin 3 mg/kg s.c.	5	>130.8±11.7	>150	>216.9±49.4	37.0±8.0	81.0±7.0	0.16±0.02
Heparin 3 mg/kg s.c. with Protamine 3 mg/kg i.v.	5	80.1±9.0	27.5±2.0	42.3±7.3	1.8±1.7	3.6±2.0	0.08±0.07
CY 216 3 mg/kg s.c.	5	89.2±18.4	>150	67.7±18.2	40.3±8.9	51.3±4.5	0.18±0.04
CY 216 3 mg/kg s.c. with Protamine 3 mg/kg i.v.	5	71.7±16.7	31.0±3.1	62.9±12.5	28.0±9.6	4.5±2.6	0.09±0.03

Data represents a mean ± S.E.

APPENDIX XXV

APPENDIX XXVa

EX VIVO ACTIVITY AND CLOT SCORES AFTER PROTAMINE ADMINISTRATION TO RABBITS

Time: Pre-challenge

Treatment	N	APTT (seconds)	TT 5U (seconds)	Heptest (seconds)	Anti-Xa (% Inhibition)	Anti-IIa % Inhibition	Clot Score
Control	5	83.9±1.0	32.0±4.1	30.8±3.8	0	0	3.1±0.3
Protamine 100 μg/kg i.v.	5	102.3±9.0	35.3±3.3	28.7±4.2	0	0	2.8±0.3
Protamine 500 μg/kg i.v.	5	83.7	72.6	37.7	7.5	10	2.3±0.2

Data represents a mean ± S.E.

APPENDIX XXVb

EX VIVO ACTIVITY AND CLOT SCORES AFTER HEPARIN AND PROTAMINE ADMINISTRATION TO RABBITS

Time: Pre-challenge

Treatment	N	APTT (seconds)	TT 5U (seconds)	Heptest (seconds)	Anti-Xa (% Inhibition)	Anti-IIa % Inhibition	Clot Score
Heparin 25 μg/kg i.v.	5	124.7±13.2	46.7±4.4	44.9±9.7	4.8±2.9	2.0±0.9	1.4±0.2
Heparin 50 μg/kg i.v.	5	111.2±17.2	132.3±57.1	76.8±10.6	5.0± 2.1	14.3±8.0	0.5±0.2
Heparin 100 μg/kg i.v.	5	146.8±29.7	>150±0	128.9±27.7	25.0±10.4	32.4±9.0	0.6±0.3
Heparin 100 μg/kg i.v. with Protamine 100 μg/kg i.v.	5	57.6±10.2	40.3±5.2	37.2±6.8	2.6±1.0	1.25±2.5	2.7±0.3
Heparin 100 μg/kg i.v. with Protamine 200 μg/kg i.v.	5	122.9±12.3	37.5±1.9	47.0±2.0	2.4±2.4	12.4±3.9	2.9±0.3

Data represents a mean ± S.E.

APPENDIX XXVc

EX VIVO ACTIVITY AND CLOT SCORES AFTER CY 216 AND PROTAMINE ADMINISTRATION TO RABBITS

Time: Pre-challenge

Treatment	N	APTT (seconds)	TT 5U (seconds)	Heptest (seconds)	Anti-Xa (% Inhibition)	Anti-IIa % Inhibition	Clot Score
CY 216 25 μg/kg i.v.	5	58.0±6.4	37.0±1.6	42.4±4.1	1.8±1.2	1.2±1.2	2.2±0.5
CY 216 50 μg/kg i.v.	5	49.1±14.4	50.7±9.3	42.1±2.1	5.0±4.6	1.3±1.4	1.5±0.2
CY 216 100 μg/kg i.v.	5	67.7±12.9	49.1±10.7	63.6±11.8	12.8±8.7	5.8±4.5	0.4±0.2
CY 216 100 μg/kg i.v. with Protamine 100 μg/kg i.v.	5	58.0±13.6	34.1±3.4	46.2±4.1	8.8±2.8	7.4±1.9	1.7±0.4
CY 216 100 μg/kg i.v. with Protamine 200 μg/kg i.v.	5	113±6.6	38.4±3.1	43.4±1.3	9.2±2.4	6.4±2.8	2.2±0.2

Data represents a mean ± S.E.

APPENDIX XXVd

EX VIVO ACTIVITY AND CLOT SCORES AFTER HEPARIN AND CY 216 AND PROTAMINE ADMINISTRATION TO RABBITS

Time: Pre-challenge

Treatment	N	APTT (seconds)	TT 5U (seconds)	Heptest (seconds)	Anti-Xa (% Inhibition)	Anti-IIa % Inhibition	Clot Score
Heparin 1 mg/kg s.c.	5	61.0±10.5	128.8±13.4	62.4±12.9	15.8±4.5	16.2±3.9	1.5±0.4
Heparin 1 mg/kg s.c. with protamine 1 mg/kg i.v.	5	78.8±18.7	85.9±25.1	39.5±5.7	12.8±3.9	18.5±9.4	2.8±0.4
CY 216 1 mg/kg s.c.	5	95.4±4.4	54.8±8.9	42.7±12.6	22.5±6.4	7.0±1.9	1.9±0.4
CY 216 1 mg/kg s.c. with Protamine 1 mg/kg i.v.	5	73.7±12.0	37.8±5.2	58.4±10.9	15.0±2.9	9.8±2.5	2.0±0.4

Data represents a mean ± S.E.

APPENDIX XXVI

APPENDIX XXVIa

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF HEPARIN IN PRIMATES

Dose: 0.7 mg/kg i.v.

Time (minutes)	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^{R} (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
0	24.4 ± 1.3	28.1 ± 0	25.4 ± 2.3	0 ± 0	0 ± 0
5	>150 ± 0	>150 ± 0	>300 ± 0	92.0 ± 2.2	90.6 ± 2.9
30	>150 ± 0	>150 ± 0	269.4 ± 30.5	83.4 ± 8.1	87.8 ± 2.0
60	129.4 ± 10.4	>150 ± 23.4	178.7 ± 24.3	77.0 ± 4.8	85.0 ± 2.5
180	33.5 ± 6.1	80.2 ± 6.0	55.3 ± 6.0	17.0 ± 6.1	28.4 ± 7.4
360	25.9 ± 1.6	25.9 ± 1.2	23.0 ± 1.2	4.2 ± 2.4	9.2 ± 4.1

Data represents a mean ± S.E., N=4.

APPENDIX XXVIb

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF HEPARIN AND PROTAMINE SULFATE IN PRIMATES

Heparin dose: 0.7 mg/kg i.v.
Protamine dose: 0.7 mg/kg i.v.

Time (minutes)	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^{R} (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
0	36.7 ± 5.3	19.8 ± 3.1	20.2 ± 1.4	0 ± 0	0 ± 0
5	>150 ± 0	>150 ± 0	>300 ± 0	97.3 ± 1.8	94.3 ± 0.5
15	86.8 ± 18.0	89.2 ± 11.2	79.9 ± 2.9	114.0 ± 6.7	49.8 ± 8.5
30	56.1 ± 7.5	73.9 ± 7.5	67.6 ± 3.0	45.8 ± 12.8	35.0 ± 3.0
60	55.1 ± 10.7	25.5 ± 3.8	63.7 ± 2.0	26.8 ± 4.6	31.6 ± 4.3
180	46.8 ± 7.7	16.3 ± 2.0	24.8 ± 0.7	6.0 ± 3.3	5.1 ± 2.3
360	23.6 ± 1.3	17.0 ± 1.5	26.1 ± 0.3	4.8 ± 2.0	0.8 ± 0.8

Data represents a mean ± S.E., N=5.

Protamine was administered immediately after the 5 minute blood draw.

APPENDIX XXVIc

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF HEPARIN IN PRIMATES

Heparin dose: 0.7 mg/kg i.v.
Protamine dose: 1.4 mg/kg i.v.

Time (minutes)	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^{R} (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
0	25.1 ± 2.5	17.2 ± 0.8	26.5 ± 2.0	0 ± 0	0 ± 0
5	>150	>150 ± 0	>300 ± 0	79.6 ± 4.7	94.4 ± 1.3
15	34.1 ± 3.6	68.6 ± 40.8	60.4 ± 5.5	58.6 ± 6.1	26.4 ± 7.7
30	28.6 ± 1.9	26.7 ± 1.5	60.3 ± 1.6	49.3 ± 7.9	40.6 ± 6.6
60	28.0 ± 2.9	22.5 ± 0.4	49.0 ± 2.2	50.3 ± 12.1	28.0 ± 2.1
180	24.7 ± 1.6	21.4 ± 0.8	29.0 ± 0.8	21.3 ± 1.2	5.6 ± 3.6
360	25.0 ± 2.1	18.8 ± 0.8	20.5 ± 0.8	4.8 ± 2.4	2.4 ± 2.4

Data represents a mean ± S.E., N=5.

Protamine was administered immediately after the 5 minute blood draw.

APPENDIX XXVId

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF HEPARIN IN PRIMATES

Heparin dose: 0.7 mg/kg i.v.
Protamine dose: 2.1 mg/kg i.v.

Time (minutes)	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest® (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
0	25.2 ± 1.0	25.0 ± 1.4	18.4 ± 1.5	0 ± 0	0 ± 0
5	>150 ± 0	>150 ± 0	234.1 ± 46.5	90.5 ± 2.6	90.3 ± 4.3
15	28.2 ± 1.0	50.6 ± 10.4	37.0 ± 1.4	52.8 ± 12.4	16.8 ± 2.8
30	30.0 ± 0	94.0 ± 12.2	28.5 ± 0.5	15.0 ± 0.9	18.0 ± 0.8
60	25.8 ± 1.0	63.5 ± 7.5	29.7 ± 5.2	24.3 ± 11.5	4.3 ± 3.4
180	23.6 ± 1.4	37.0 ± 3.6	25.6 ± 1.7	14.2 ± 8.0	7.6 ± 2.6
360	15.4 ± 2.0	23.3 ± 2.5	21.9 ± 1.1	7.3 ± 5.9	6.7 ± 5.3

Data represents a mean ± S.E., N=5.

Protamine was administered immediately after the 5 minutes blood draw.

APPENDIX XXVIe

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF CY 216 IN PRIMATES

Dose: 0.7 mg/kg i.v.

Time (minutes)	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^{R} (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
0	24.6 ± 2.3	28.4 ± 3.9	31.3 ± 1.7	0 ± 0	0
5	129.4 ± 20.7	>150 ± 0	>300 ± 0	91.0 ± 2.4	68.2 ± 7.9
30	39.4 ± 3.9	121.8 ± 16.5	120.4 ± 20.4	57.3 ± 9.4	39.0 ± 10.0
60	33.9 ± 2.3	110.7 ± 24.0	92.1 ± 8.9	49.2 ± 5.0	27.8 ± 8.8
180	26.1 ± 1.4	31.7 ± 3.8	55.4 ± 3.2	19.2 ± 5.4	8.4 ± 3.4
360	23.8 ± 1.4	30.2 ± 6.0	38.7 ± 3.8	3.4 ± 1.6	4.0 ± 0.0

Data represents a mean ± S.E., N=4.

APPENDIX XXVIf

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF CY 216 AND PROTAMINE SULFATE IN PRIMATES

CY 216 dose: 0.7 mg/kg i.v.
Protamine dose: 0.7 mg/kg i.v.

Time (minutes)	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^{R} (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
0	25.0 ± 2.6	21.4 ± 2.5	21.4 ± 1.2	0 ± 0	0 ± 0
5	98.6 ± 29.9	>150 ± 0	>300 ± 0	93.6 ± 3.0	81.6 ± 3.8
15	37.4 ± 4.1	31.8 ± 4.8	126.1 ± 14.1	68.4 ± 7.8	21.8 ± 9.0
30	34.5 ± 3.3	29.7 ± 2.4	132.4 ± 19.0	77.8 ± 5.2	41.8 ± 7.1
60	34.2 ± 2.1	35.5 ± 4.2	92.9 ± 2.4	67.3 ± 7.8	41.3 ± 6.0
180	28.0 ± 1.2	18.9 ± 2.3	60.2 ± 3.1	42.6 ± 8.1	6.5 ± 2.0
360	24.6 ± 3.4	20.4 ± 1.3	39.5 ± 0.9	22.8 ± 4.8	8.0 ± 6.2

Data represents a mean ± S.E., N=5.

Protamine was administered immediately after the 5 minute blood draw.

APPENDIX XXVIg

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF CY 216 AND PROTAMINE SULFATE IN PRIMATES

CY 216 dose: 0.7 mg/kg i.v.
Protamine dose: 1.4 mg/kg i.v.

Time (minutes)	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^{R} (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
0	30.5 ± 3.5	19.0 ± 0.90	21.0 ± 0	0 ± 0	0 ± 0
5	>150	135.9 ± 6.3	>300 ± 0	71.3 ± 3.1	76.6 ± 6.1
15	43.4 ± 8.1	65.1 ± 13.9	109.0 ± 7.8	44.5 ± 6.2	33.3 ± 3.6
30	50.4 ± 10.0	72.6 ± 13.5	95.5 ± 5.2	37.5 ± 3.9	20.0 ± 11.5
60	39.6 ± 6.2	27.9 ± 3.3	83.9 ± 7.8	26.0 ± 10.0	21.7 ± 11.0
180	35.2 ± 6.1	15.0 ± 0.1	55.1 ± 8.1	11.3 ± 2.1	5.3 ± 3.0
360	45.8 ± 2.0	15.2 ± 0.3	39.8 ± 4.8	5.0 ± 5.0	2.5 ± 1.4

Data represents a mean ± S.E., N=5.

Protamine was administered immediately after the 5 minute blood draw.

APPENDIX XXVIh

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF CY 216 AND PROTAMINE SULFATE IN PRIMATES

CY 216 dose: 0.7 mg/kg i.v.
Protamine dose: 2.1 mg/kg i.v.

Time (minutes)	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^{R} (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
0	25.5 ± 0.8	24.8 ± 2.8	20.6 ± 1.1	0 ± 0	0 ± 0
5	63.6 ± 13.0	>150 ± 0	288.0 ± 11.3	98.8 ± 0.2	70.7 ± 4.6
15	34.7 ± 1.3	67.3 ± 15.0	95.5 ± 7.3	35.8 ± 7.3	33.3 ± 12.7
30	37.2 ± 3.4	20.8 ± 0	117.8 ± 6.0	25.6 ± 5.2	27.8 ± 11.7
60	35.1 ± 3.9	24.1 ± 1.2	83.2 ± 1.2	18.6 ± 2.2	10.5 ± 4.1
180	27.5 ± 1.2	17.7 ± 0.5	61.0 ± 2.9	12.4 ± 4.2	10.3 ± 10.5
360	24.6 ± 1.6	21.6 ± 0.3	49.9 ± 1.4	5.8 ± 2.7	7.7 ± 3.3

Data represents a mean ± S.E., N=5.

Protamine was administered immediately after the 5 minute blood draw.

APPENDIX XXVIi

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF HEPARIN IN PRIMATES

Dose: 0.7 mg/kg s.c.

Time (minutes)	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^{R} (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
0	31.8 ± 3.2	25.4 ± 2.7	21.6 ± 0.7	0 ± 0	0 ± 0
120	34.7 ± 2.5	37.8 ± 8.0	49.8 ± 3.0	8.2 ± 2.6	5.8 ± 3.7
240	35.5 ± 2.9	29.7 ± 4.6	44.6 ± 4.1	7.3 ± 2.7	7.0 ± 3.3
360	30.6 ± 3.3	27.5 ± 3.4	31.0 ± 2.2	3.8 ± 1.2	4.4 ± 2.3
540	29.0 ± 3.1	25.6 ± 2.6	22.4 ± 1.2	4.4 ± 2.4	1.4 ± 1.4
720	29.7 ± 3.1	24.9 ± 2.2	24.1 ± 1.4	3.6 ± 0.7	0 ± 0

Data represents a mean ± S.E., N=5.

APPENDIX XXVIj

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF HEPARIN WITH PROTAMINE SULFATE IN PRIMATES

Heparin dose: 0.7 mg/kg s.c.
Protamine dose: 0.7 mg/kg i.v.

Time (minutes)	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^{R} (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
0	25.1 ± 1.0	26.4 ± 0.5	26.0 ± 1.2	0 ± 0	0 ± 0
120	30.2 ± 2.8	30.8 ± 2.4	38.3 ± 5.7	7.8 ± 3.2	7.4 ± 3.5
240	26.5 ± 2.2	31.7 ± 1.6	40.2 ± 5.8	8.4 ± 2.1	8.4 ± 2.7
245	26.4 ± 2.4	37.3 ± 10.9	26.9 ± 1.8	7.6 ± 3.1	4.6 ± 2.7
540	25.1 ± 1.9	26.1 ± 0.9	24.5 ± 1.4	4.4 ± 1.6	3.8 ± 3.8
720	22.3 ± 1.3	28.4 ± 1.3	21.2 ± 1.1	7.4 ± 3.3	2.4 ± 1.6

Data represents a mean ± S.E., N=5.

Protamine was administered immediately after the 240 minute blood draw.

APPENDIX XXVIk

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF CY 216 IN PRIMATES

Dose: 0.7 mg/kg s.c.

Time (minutes)	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^{R} (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
0	25.54 ± 0.8	27.8 ± 1.1	20.7 ± 0.9	0 ± 0	0 ± 0
120	29.7 ± 1.2	34.8 ± 1.9	62.2 ± 3.1	32.2 ± 4.9	9.2 ± 3.2
240	30.0 ± 1.2	38.8 ± 1.7	60.2 ± 2.0	53.0 ± 5.2	13.3 ± 5.2
360	28.8 ± 1.2	19.9 ± 0.6	46.3 ± 2.6	27.8 ± 6.0	6.6 ± 2.7
540	27.4 ± 1.0	21.6 ± 2.1	37.3 ± 2.1	18.8 ± 3.5	12.3 ± 2.7
720	28.6 ± 1.5	24.3 ± 0.9	36.0 ± 2.8	14.8 ± 5.3	4.8 ± 2.0

Data represents a mean ± S.E., N=5.

APPENDIX XXVI1

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF CY 216 WITH PROTAMINE SULFATE IN PRIMATES

CY 216 Dose: 0.7 mg/kg s.c.
Protamine Dose: 0.7 mg/kg i.v.

Time (minutes)	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^{R} (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
0	26.5 ± 1.5	26.7 ± 1.6	23.8 ± 1.4	0 ± 0	0 ± 0
120	29.9 ± 2.4	32.8 ± 2.2	70.9 ± 1.9	31.5 ± 2.8	7.6 ± 3.2
240	29.5 ± 1.3	29.9 ± 1.0	66.0 ± 6.2	31.2 ± 5.0	5.4 ± 2.4
245	33.9 ± 3.3	30.6 ± 3.4	65.8 ± 5.0	26.8 ± 2.2	0 ± 0
540	29.8 ± 1.3	28.1 ± 1.5	58.8 ± 4.4	29.0 ± 2.5	5.4 ± 1.5
720	29.9 ± 2.0	22.9 ± 3.6	36.4 ± 3.8	19.4 ± 2.1	3.6 ± 1.0

Data represents a mean ± S.E., N=5.

Protamine was administered immediately after the 240 minute blood draw.

APPENDIX XXVIm

TIME COURSE OF THE PHARMACODYNAMIC ACTIVITY OF CY 216 WITH PROTAMINE SULFATE IN PRIMATES

CY 216 Dose: 0.7 mg/kg s.c.
Protamine Dose: 1.4 mg/kg i.v.

Time (minutes)	APTT (seconds)	Thrombin Time (5 U/ml) (seconds)	Heptest^{R} (seconds)	Anti-Xa (% inhibition)	Anti-IIa (% inhibition)
0	24.7 ± 0.9	22.5 ± 0.8	20.0 ± 1.1	0 ± 0	0 ± 0
120	27.8 ± 1.3	23.9 ± 2.4	58.9 ± 2.7	40.3 ± 4.5	12.5 ± 0.9
240	27.4 ± 1.6	29.1 ± 2.6	60.9 ± 3.0	42.5 ± 4.3	11.0 ± 1.5
245	30.0 ± 3.0	37.0 ± 9.2	47.6 ± 9.0	35.8 ± 1.3	2.8 ± 1.7
540	25.6 ± 1.5	29.7 ± 2.6	52.7 ± 1.2	27.5 ± 5.8	2.0 ± 2.0
360	27.6 ± 1.5	28.5 ± 1.6	41.9 ± 3.2	9.0 ± 3.7	0 ± 0

Data represents a mean ± S.E., N=5.

Protamine was administered immediately after the 240 minutes blood draw.

APPENDIX XXVII

COMPARISON OF HEPARIN AND CY 216 SUPPLEMENTED TO VARIOUS PLASMA PREPARATIONS

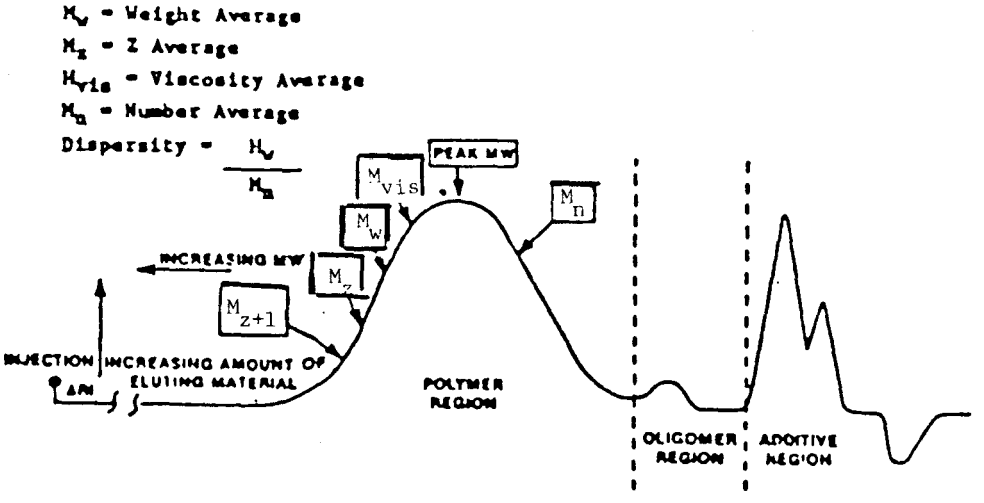
Plasma Preparation	Heparin (μg/ml)	CY 216 (μg/ml)
Test: APTT		
NHP	1.3	>10
NMP	1.4	>10
NRP	1.9	>10
NratP	3.8	>10
Test: Heptest		
NHP	.82	1.3
NMP	.82	1.3
NRP	6.2	1.3
NratP	2.05	7.5
Test: Anti-Xa		
NHP	2.5	6.0
NMP	2.5	5.0
NRP	3.8	9.0
NratP	4.0	7.2
Test: Anti-IIa		
NHP	1.5	8.2
NMP	1.3	4.6
MRP	3.3	10.0
NratP	2.5	7.4

Heparin or CY 216 was supplemented to NHP, NMP, NRP and NratP in a concentration range of 0-10 μg/ml. For the clotting tests the concentration of heparin or CY 216 at which the baseline was doubled is reported. For the amidolytic assays, the concentration which produced 50% inhibition was reported.

APPENDIX XXVIII

APPENDIX XXVIII

MOLECULAR PARAMETERS OF A HETEROGENEOUS GLYCOSAMINOGLYCAN *



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

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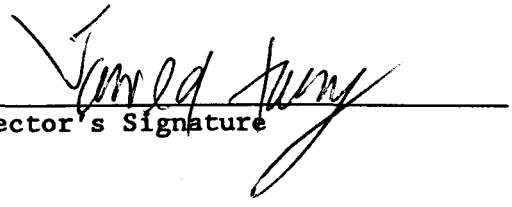
The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

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

10/23/89
Date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