1995

Pharmacokinetics and Pharmacodynamics of Recombinant Hirudin Variant 2 (RHV2) in Animal Models

Lalitha Iyer
Loyola University Chicago

LOYOLA UNIVERSITY CHICAGO

PHARMACOKINETICS AND PHARMACODYNAMICS OF RECOMBINANT HIRUDIN VARIANT 2 (rHV2) IN ANIMAL MODELS

VOLUME I
(CHAPTERS I, II, III, IV)

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN PARTIAL FULFILLMENT OF THE REQUIREMENT

FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY AND
EXPERIMENTAL THERAPEUTICS

BY

LALITHA IYER

CHICAGO, ILLINOIS

MAY, 1995

ACKNOWLEDGEMENTS

First of all, I would like to thank Dr. Jawed Fareed for being a constant source of inspiration, guidance and support. I would like to express my sincere gratitude to Dr. Fareed for having provided me with state-of-the-art facilities to work toward my dissertation and for giving me the opportunity to interact with several renowned scientists in my area of research interest. I am grateful to Dr. Israel Hanin for being extremely supportive during all stages of my graduate career at Loyola. I would like to thank Dr. David Luke for being a constant source of information and guidance. I sincerely appreciate the help and guidance given by the remaining members of my dissertation committee: Drs. Edward Bermes, Jr., Stanley Lorens, and Harry Messmore. I am grateful to the department of Pharmacology for providing me with resources to complete my dissertation. I would like to thank Dr. Ralph Leischner and the department of Pathology, for providing me with funds, resources and laboratory space.

I would like to thank Dr. Jeanine Walenga for her guidance and encouragement throughout my graduate work. I am grateful to Walter Jeske and the graduate students in Pharmacology for their friendship and support. I sincerely appreciate the technical assistance and support offered to me

by the staff of Hemostasis Research Laboratories: Scott Asselmeier, Dr. Peter Bacher, Bob Calabria, Elayne Grzeda, Tony Fasanella, Dr. Kaiding Fu, Sally Healey, Debra Hoppensteadt, Dr. Omer Iqbal, Yelena Khenkina, Mike Koza, Dr. Armen Ovsepian, Jordan Shavit and Dr. Li-Hui Yang. I would like to thank Dr. Bermes and the Clinical Laboratory Division for providing me with laboratory profiles on the samples from animal studies. I acknowledge the assistance given by the staff of Comparative Medicine Facility in the handling and care of animals, and the staff of Biomedical Media Services in the preparation of some of the illustrations in this dissertation.

I am forever indebted to my mother who inculcated the values of knowledge and education in me. Special thanks to Pravin, Geetha, Raji, Daisy and Vinny, for keeping my spirits high. Lastly, but certainly not the least, I would like to thank my husband, Shankar, without whose love, patience and support, this work would not have been possible.

To Shankar

ABSTRACT

Lalitha Iyer

Loyola University Chicago

PHARMACOKINETICS AND PHARMACODYNAMICS OF RECOMBINANT HIRUDIN VARIANT 2 (rHV2) IN ANIMAL MODELS

Hirudin is reportedly the most potent and specific natural inhibitor of thrombin (II_a), with an inhibitory constant (K_i) in the picomolar range. This 65/66 amino acid polypeptide, extracted from the medicinal leech, *Hirudo medicinalis*, was used in ancient medicine as an anticoagulant. More recently, the development and production of recombinant forms of hirudin variants have brought hirudin back into the focus of research interest.

The primary objective of this dissertation was to investigate the relationship between pharmacokinetics (PK) and pharmacodynamics (PD) of recombinant hirudin variant 2 (rHV2) in valid animal models. To accomplish this, anticoagulant, antithrombin and enzyme-linked immunosorbent assay (ELISA) methods were developed and optimized. The PK and PD analysis of rHV2 was performed in three animal models: rats, rabbits and dogs, using two routes of administration: i.v. (bolus) and s.c.

A polyclonal antibody based competitive ELISA method was

experimentally validated and used to measure absolute concentrations of rHV2 in plasma. This enabled the determination of true pharmacokinetic characteristics of rHV2. The *in vitro* and *ex vivo* pharmacodynamics of rHV2 were studied using coagulation tests such as activated partial thromboplastin time (APTT), thrombin time (TT) and calcium thrombin time (Ca^{+2}TT), and an amidolytic antithrombin assay. The *in vivo* antithrombotic and hemorrhagic profile of rHV2 were studied using a modified Wessler's jugular vein stasis thrombosis model and ear blood loss model in rabbits, respectively. Renal function status after rHV2 administration was evaluated using ^{3}H-inulin clearance analysis in rats. In addition, clinical laboratory profile was obtained after rHV2 administration in dogs and rats.

Utilizing a newly developed thrombin titration method, the potency of rHV2 was determined to be $\approx$ 15,873 antithrombin units/mg. Species and assay dependent antithrombin effects were observed in the concentration-response studies after *in vitro* supplementation of rHV2.

The observations from this dissertation revealed that rHV2 produced dose dependent antithrombotic and bleeding effects in rabbits, with a broad therapeutic window. In the pharmacokinetic studies, rHV2 exhibited a terminal elimination half-life of $\approx$ 45 to 60 minutes after i.v. administration. The biological residence time of rHV2 was increased to $\approx$ 3 hours after s.c. administration. Significant extravascular distribution of rHV2 was evident after both routes of administration, in all three species. rHV2 exhibited linear

pharmacokinetics after escalating i.v. and s.c. administration in rats and dogs.

In the PK/PD studies, it was found that rHV2 displayed a dose, time and route dependent *ex vivo* antithrombin profile. The time course of *ex vivo* antithrombin activity of rHV2 paralleled the time course of plasma rHV2 concentrations, indicating a direct relationship between PK and PD of this agent, in all three animal models.

Repeated, daily administration of rHV2 in dogs did not produce any major accumulation of rHV2, or attenuation of anticoagulant activity. rHV2 did not produce renal compromise in rats after both routes of administration. Furthermore, there were no significant fluctuations in clinical laboratory profile after single dose administration in rats and multiple dose administration in dogs.

These studies indicate that rHV2 is a potent anticoagulant and antithrombotic agent with a predictable pharmacokinetic/pharmacodynamic profile, and exhibits a desirable therapeutic index.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS iii

ABSTRACT v

LIST OF FIGURES xiii

LIST OF TABLES xxi

LIST OF ABBREVIATIONS xxiv

VOLUME I

Chapter

I. REVIEW OF LITERATURE 1

History of Hirudin 1
Structural Analysis and Biotechnology of Hirudin 5
The Coagulation Cascade 12
Mechanism of Antithrombin Activity of Hirudin:
The Hirudin-Thrombin Interaction 21
Laboratory Assays for Hirudin 27
Pharmacokinetics of Hirudin 29
Comparison of Hirudin with Heparin 33
Neutralization of Recombinant Hirudin 36
Potential Clinical Use of Recombinant Hirudin 37
Hirudin Related Agents 42
Other Site-Directed Antithrombotic Agents 44

II. STATEMENT OF PURPOSE 46

III. MATERIALS, METHODS AND EXPERIMENTAL PROTOCOLS 50

Materials 50

Recombinant Hirudin (rH) 50
Thrombin 51
Reagents and Kits 51
Major Instruments 52

Chapter Page

Animals 53

Methods 56
Global Clot-based Assays 56
Δ 215-225 Method for Protein Determination 58
Chromogenic Anti-IIa Assay 58
Enzyme-Linked Immunosorbent Assay (ELISA) 60
Rabbit Jugular Vein Stasis Thrombosis Model 62
Rabbit Ear Bleeding Model 67

Experimental Protocols 73

In Vitro Studies 73

1. Construction of Concentration-Response Curves after *In Vitro* Supplementation of rHV2 in Plasma Systems 73
2. Determination of Specific Activity of rHV2 using a Thrombin Titration Method 74
3. Determination of Protein Content of rHV2 by Δ 215-225 Method 76

In Vivo Studies 76

1. Assessment of Antithrombotic Profile of rHV2 using a Modified Jugular Vein Stasis Thrombosis Model in Rabbits - A Dose-Ranging Study 76
2. Assessment of Hemorrhagic Activity of rHV2 using a Modified Rabbit Ear Blood Loss Model 77
3. Evaluation of Pharmacokinetics and Time Course of Antithrombotic Activity after rHV2 Administration in Rabbits 78
4. Determination of Pharmacokinetic Characteristics and *Ex Vivo* Antithrombin Activity of rHV2 in Rats 80

5. Evaluation of Renal Function after rHV2 Administration in Rats 82
6. Investigation of Pharmacokinetics and Pharmacodynamics of rHV2 after Single Dose Administration in Dogs 83
7. Investigation of Pharmacodynamics of rHV2 after Repeated Administration in Dogs 85

Data Analysis 86

Statistical Analysis 88

Approval of Protocols 89

IV. RESULTS 91

In Vitro Studies 91

1. Construction of Concentration-Response Curves after *In Vitro* Supplementation of rHV2 in Plasma Systems 91
2. Determination of Specific Activity of rHV2 using a Thrombin Titration Method 103
3. Determination of Protein Content of rHV2 using Δ 215-225 Method 106

In Vivo Studies 106

1. Assessment of Antithrombotic Profile of rHV2 using a Modified Jugular Vein Stasis Thrombosis Model in Rabbits - A Dose-Ranging Study 106
2. Assessment of Hemorrhagic Activity of rHV2 using a Modified Rabbit Ear Blood Loss Model . . . 124
3. Evaluation of Pharmacokinetics and Time Course of Antithrombotic Activity after rHV2 Administration in Rabbits 128
4. Determination of Pharmacokinetic Characteristics and *Ex Vivo* Antithrombin Activity of rHV2 in Rats 156

5. Evaluation of Renal Function after rHV2 Administration in Rats 165
a. Serum Chemistry Profile in Rats after Administration of rHV2 169
b. Hematological Profile in Rats after Administration of rHV2 175
6. Investigation of Pharmacokinetics and Pharmacodynamics of rHV2 after single Dose Administration in Dogs 179
7. Investigation of Pharmacodynamics of rHV2 after Repeated Administration in Dogs 202
a. Serum Chemistry Profile in Rats after Administration of rHV2 215
b. Hematological Profile in Rats after Administration of rHV2 230

VOLUME II

V. DISCUSSION 239

In Vitro Studies 239

1. Construction of Concentration-Response Curves after *In Vitro* Supplementation of rHV2 in Plasma Systems 240
2. Determination of Specific Activity of rHV2 using a Thrombin Titration Method 249
3. Determination of Protein Content of rHV2 using Δ 215-225 Method 250

In Vivo Studies 251

1. Assessment of Antithrombotic Profile of rHV2 using a Modified Jugular Vein Stasis Thrombosis Model in Rabbits - A Dose-Ranging Study 250
2. Assessment of Hemorrhagic Activity of rHV2 using a Modified Rabbit Ear Blood Loss Model . . . 261
3. Evaluation of Pharmacokinetics and Time Course of Antithrombotic Activity after rHV2 Administration in Rabbits 264

Chapter Page

4. Determination of Pharmacokinetic Characteristics and *Ex Vivo* Antithrombin Activity of rHV2 in Rats 275
5. Evaluation of Renal Function after rHV2 Administration in Rats 278
   a. Serum Chemistry Profile in Rats after Administration of rHV2 280
   b. Hematological Profile in Rats after Administration of rHV2 284
6. Investigation of Pharmacokinetics and Pharmacodynamics of rHV2 after single Dose Administration in Dogs 286
7. Investigation of Pharmacodynamics of rHV2 after Repeated Administration in Dogs 290
   a. Serum Chemistry Profile in Rats after Administration of rHV2 292
   b. Hematological Profile in Rats after Administration of rHV2 294

VI. SUMMARY 295

VII. CONCLUSIONS 302

Appendix

I. PHARMACOKINETIC SURVEY OF NATURAL AND RECOMBINANT HIRUDIN 304

II. SPECIFICATIONS ON rHV2 (Batch RHE 15, Sanofi Reserche, France) 311

III. COPYRIGHT PERMISSION FORMS 318

REFERENCES 341

VITA 364

LIST OF FIGURES

Figure		Page
1.	Use of leeches in ancient medicine	2
2.	Amino acid sequences of hirudin variants 1, 2 and 3 (HV1, HV2, HV3)	8
3.	A comparison of structural sequences of natural hirudin and recombinant hirudin variant 2 (Lys 47)	9
4.	Primary structure of recombinant hirudin variant 2 (rHV2)	11
5.	The Coagulation Cascade	15
6.	Scheme of hirudin-thrombin interaction	24
7.	Scheme of competitive ELISA method for rH (part I)	63
8.	Scheme of competitive ELISA method for rH (part II)	64
9.	An illustration of the modiified Wessler's rabbit jugular vein stasis thrombosis model	65
10.	Clot-score grading system in the rabbit jugular vein stasis thrombosis model	68
11.	An illustration of the modified rabbit ear blood loss model	71
12.	*In vitro* calibration curves using thrombin time and calcium thrombin time after supplementation of rHV2 in normal rabbit plasma (NRP).	93
13.	*In vitro* antithrombin profile of rHV2 supplemented in normal rabbit plasma (NRP).	95

14. *In vitro* antithrombin profile of rHV2 supplemented in normal dog plasma (NDP). 98

15. *In vitro* antithrombin profile of rHV2 supplemented in blood bank plasma (BBP). 100

16. *In vitro* standard curve for chromogenic anti-IIa assay after supplementation of rHV2 in normal rat plasma (NRatP). 102

17. Thrombin titration curve for determination of specific activity of rHV2. 105

18. *In vivo* antithrombotic activity of rHV2 after i.v. administration in rabbits 107

19. *Ex vivo* antithrombin activity of rHV2 using APTT (OT), after i.v. administration in rabbits 109

20. *Ex vivo* antithrombin activity of rHV2 using APTT (Dade), after i.v. administration in rabbits 111

21. *Ex vivo* antithrombin activity of rHV2 using TT (10 U/mL), after i.v. administration in rabbits. 112

22. *Ex vivo* antithrombin activity of rHV2 using Ca^{+2}TT (10 U/mL), after i.v. administration in rabbits. 113

23. *Ex vivo* antithrombin activity of rHV2 using chromogenic anti-IIa assay, after i.v. administration in rabbits. 115

24. *In vivo* antithrombotic activity of rHV2 after s.c. administration in rabbits 117

25. *Ex vivo* antithrombin activity of rHV2 using APTT (OT), after s.c. administration in rabbits. 119

26. *Ex vivo* antithrombin activity of rHV2 using APTT (Dade), after s.c. administration in rabbits. 120

27. *Ex vivo* antithrombin activity of rHV2 using TT (10 U/mL), after s.c. administration in rabbits. 121

28. *Ex vivo* antithrombin activity of rHV2 using $Ca^{+2}TT$ (10 U/mL), after s.c. administration in rabbits 122

29. *Ex vivo* antithrombin activity of rHV2 using chromogenic anti-IIa assay, after s.c. administration in rabbits 123

30. Hemorrhagic effects of rHV2 in a rabbit modified ear blood loss model after i.v. administration 125

31. Hemorrhagic effects of rHV2 in a rabbit modified ear blood loss model after s.c. administration 127

32. Time course of *in vivo* antithrombotic activity of rHV2 after i.v. administration in rabbits 130

33. Time course of *ex vivo* antithrombin activity using coagulation tests, APTT (OT) and APTT (Dade), after i.v. administration in rabbits 131

34. Time course of *ex vivo* antithrombin activity using $Ca^{+2}TT$ (10 U/mL) assay, after i.v. administration in rabbits 133

35. Time course of *ex vivo* antithrombin activity using chromogenic anti-IIa assay, after i.v. administration in rabbits 134

36. Pharmacokinetics of rHV2 after i.v. administration in rabbits. 135

37. Correlation between plasma concentrations of rHV2 and *in vivo* antithrombotic activity after i.v. administration in rabbits 139

38. Relationship between plasma concentrations of rHV2 and *ex vivo* antithrombin activity using APTT tests and anti-IIa assay, after i.v. administration in rabbits. 140

39. Correlation between plasma concentrations of rHV2 and *ex vivo* antithrombin activity using $Ca^{+2}TT$ (10 U/mL), after i.v. administration in rabbits. 142

40. Time-course of *in vivo* antithrombotic activity of rHV2 after s.c. administration in rabbits 143

41. Time course of *ex vivo* antithrombin activity of rHV2 using APTT tests, after s.c. administration in rabbits 144

42. Time course of *ex vivo* antithrombin activity of rHV2 using Ca^{+2}TT (10 U/mL), after s.c. administration in rabbits. 146

43. Time course of *ex vivo* antithrombin activity of rHV2 using chromogenic anti-IIa assay, after s.c. administration in rabbits. 147

44. Pharmacokinetics of rHV2 after s.c. administration in rabbits 148

45. Relationship between plasma concentrations of rHV2 and *in vivo* anti-thrombotic activity, after s.c. administration in rabbits. 151

46. Relationship between plasma concentrations of rHV2 and coagulation tests, APTT (OT) and APTT (Dade), after s.c. administration in rabbits 153

47. Correlation between plasma concentrations of rHV2 and coagulation test, Ca^{+2}TT (10 U/mL), after s.c. administration in rabbits 154

48. Correlation between plasma concentrations of rHV2 and *ex vivo* anti-thrombin activity using chromogenic anti-IIa assay, after s.c. administration in rabbits 155

49. Pharmacokinetics of rHV2 after i.v. administration of increasing doses in rats 157

50. Time course of *ex vivo* antithrombin activity (chromogenic anti-IIa assay) of rHV2 in rats after i.v. administration 161

51. Pharmacokinetics of rHV2 after s.c. administration of increasing doses in rats 163

52. Time course of *ex vivo* antithrombin activity (chromogenic anti-IIa assay) of rHV2 in rats after s.c. administration 166

53. Plasma concentration time curve of ^{3}H-inulin in rats pretreated with rHV2 compared with saline treated controls 168

54. Pharmacokinetics of rHV2 after escalating i.v. bolus administration in dogs 180

55. Time-course of *ex vivo* antithrombin activity [APTT (OT)] of rHV2 after i.v. administration in dogs 185

56. Time-course of *ex vivo* antithrombin activity [APTT (Dade)] of rHV2 after i.v. administration in dogs 186

57. Time-course of *ex vivo* antithrombin activity [TT (20 U/mL)] of rHV2 after i.v. administration in dogs 187

58. Time-course of *ex vivo* antithrombin activity [Ca^{+2}TT (10 U/mL)] of rHV2 after i.v. administration in dogs . 188

59. Time-course of *ex vivo* antithrombin activity [Ca^{+2}TT (20 U/mL)] of rHV2 after i.v. administration in dogs . 189

60. Time-course of *ex vivo* antithrombin activity (chromogenic anti-IIa assay) of rHV2 after i.v. doses in dogs 190

61. Pharmacokinetics of rHV2 after escalating s.c. administration in dogs 192

62. Time-course of *ex vivo* antithrombin activity [APTT (OT)] of rHV2 after s.c. administration in dogs 196

63. Time-course of *ex vivo* antithrombin activity [APTT (Dade)] of rHV2 after s.c. administration in dogs 197

64. Time-course of *ex vivo* antithrombin activity [TT (20 U/mL)] of rHV2 after s.c. administration in dogs 198

65. Time-course of *ex vivo* antithrombin activity [Ca^{+2}TT (10 U/mL)] of rHV2 after s.c. administration in dogs . 199

66. Time-course of *ex vivo* antithrombin activity [Ca^{+2}TT (20 U/mL)] of rHV2 after s.c. administration in dogs 200

67. Time-course of *ex vivo* antithrombin activity (chromogenic anti-IIa assay) of rHV2 after s.c. administration in dogs 201

68. Time-course of plasma concentration of rHV2 after repeated i.v. administration in dogs 205

69. Time-course of *ex vivo* antithrombin activity (APTT-Dade) of rHV2 after repeated i.v. administration in dogs . . . 206

70. Time-course of *ex vivo* antithrombin activity [(Ca^{+2}TT (10 U/mL)} of rHV2 after repeated i.v. administration in dogs 207

71. Time-course of *ex vivo* antithrombin activity (chromogenic anti-IIa assay) of rHV2 after repeated i.v. administration in dogs 208

72. Time-course of plasma concentration of rHV2 after repeated s.c. administration in dogs 211

73. Time-course of *ex vivo* antithrombin activity (APTT-Dade) of rHV2 after repeated s.c. administration in dogs . . . 212

74. Time-course of *ex vivo* antithrombin activity [(Ca^{+2}TT (10 U/mL)} of rHV2 after repeated s.c. administration in dogs 213

75. Time-course of *ex vivo* antithrombin activity (chromogenic anti-IIa assay) of rHV2 after repeated s.c. administration in dogs 214

76. Serum chemistry profile after repeated i.v. administration of rHV2 in dogs (part I) 218

77. Serum chemistry profile after repeated i.v. administration of rHV2 in dogs (part II) 219

78. Serum chemistry profile after repeated i.v. administration of rHV2 in dogs (part III) 220

79. Serum chemistry profile after repeated i.v. administration of rHV2 in dogs (part IV) 221

80. Serum chemistry profile after repeated i.v. administration of rHV2 in dogs (part V) 222

81. Serum chemistry profile after repeated s.c. administration of rHV2 in dogs (part I) 225

82. Serum chemistry profile after repeated s.c. administration of rHV2 in dogs (part II) 226

83. Serum chemistry profile after repeated s.c. administration of rHV2 in dogs (part III) 227

84. Serum chemistry profile after repeated s.c. administration of rHV2 in dogs (part IV) 228

85. Serum chemistry profile after repeated s.c. administration of rHV2 in dogs (part V) 229

86. Hematological profile after repeated i.v. administration of rHV2 in dogs (part I) 232

87. Hematological profile after repeated i.v. administration of rHV2 in dogs (part II) 233

88. Hematological profile after repeated i.v. administration of rHV2 in dogs (part III) 234

89. Hematological profile after repeated s.c. administration of rHV2 in dogs (part I) 236

90. Hematological profile after repeated s.c. administration of rHV2 in dogs (part II) 237

91. Hematological profile after repeated s.c. administration of rHV2 in dogs (part III) 238

92. The sigmoidal E_{max} model describing the relationship between antithrombotic effects of rHV2 and measured plasma rHV2 concentrations in rabbits. . . . 267

LIST OF TABLES

Table		Page
1.	Laboratory assays for the determination of recombinant hirudin	28
2.	Comparison of recombinant hirudin with heparin	34
3.	Time schedule used in the modified rabbit jugular vein stasis thrombosis model	69
4.	Time schedule used in the modified rabbit ear blood loss model	72
5.	Slopes from concentration response curves after *in vitro* supplementation of rHV2 in various plasma systems	103
6.	Determination of specific activity of rHV2 using thrombin titration method	104
7.	Pharmacokinetic parameters obtained after single dose i.v. and s.c. administration of rHV2 in rabbits	137
8.	Pharmacokinetic parameters obtained after single dose i.v. administration of rHV2 in rats	159
9.	Pharmacokinetic parameters obtained after single dose s.c. administration of rHV2 in rats	164
10.	Dispositional parameters obtained in ^{3}H-inulin clearance studies in rats	169
11.	Serum chemistry profile in rats after i.v. administration of rHV2 (part I)	170

12. Serum chemistry profile in rats after i.v. administration of rHV2 (part II) 171

13. Serum chemistry profile in rats after s.c. administration of rHV2 (part I) 173

14. Serum chemistry profile in rats after s.c. administration of rHV2 (part II) 174

15. Hematological profile in rats after i.v. administration of rHV2 177

16. Hematological profile in rats after s.c. administration of rHV2 178

17. Pharmacokinetic parameters obtained after single dose i.v. administration of rHV2 in dogs 182

18. Pharmacokinetic parameters obtained after single dose s.c. administration of rHV2 in dogs 193

19. Area under the curve and clearance values after repeated administration of rHV2 in dogs 203

20. Pharmacokinetic survey on natural and recombinant hirudin (part I) 305

21. Pharmacokinetic survey on natural and recombinant hirudin (part II) 306

22. Pharmacokinetic survey on natural and recombinant hirudin (part III) 307

23. Pharmacokinetic survey on natural and recombinant hirudin (part IV) 308

24. Pharmacokinetic survey on natural and recombinant hirudin (part V) 309

25. Pharmacokinetic survey on natural and recombinant hirudin (part VI) 310

26. Amino acid composition of various recombinant hirudin preparations as determined by PICO-TAG method . . 317

LIST OF ABBREVIATIONS

Standard Abbreviations

AIDS	-	acquired immune-deficiency syndrome
AUC	-	area under the curve
AUMC	-	area under the first moment curve
b.i.d.	-	twice a day
^{o}C	-	celsius
C_{max}	-	maximum concentration
Ca^{+2}	-	calcium
$CaCl_2$	-	calcium chloride
C.D.	-	circular dichroism
cDNA	-	complementary deoxyribonucleic acid
Ci	-	curie
Cl^-	-	chloride
Cl_{ren}	-	renal clearance
Cl_{tot}	-	total clearance
cm	-	centimeter
CO_2	-	carbon dioxide

cpm	-	counts per minute
CUE	-	cumulative urinary excretion
dL	-	deciliter
DIC	-	disseminated intravascular coagulopathy
dpm	-	disintegrations per minute
E	-	extinction coefficient
EDTA	-	ethylenediaminetetraacetic acid
ELISA	-	enzyme-linked immunosorbent assay
F	-	bioavailability
fL	-	femtoliter
g	-	gram
GFR	-	glomerular filtration rate
H_2O_2	-	hydrogen peroxide
HCl	-	hydrochloric acid
HCO_3^-	-	bicarbonate
HIV	-	human immunodeficiency virus
HPLC	-	high performance liquid chromatography
i.m.	-	intramuscular
i.p.	-	intraperitoneal
IU	-	international units
i.v.	-	intravenous
IACUC	-	Institutional Animal Care and Use Committee

K^+	-	potassium
k_e	-	terminal elimination rate constant
kg	-	kilogram
L	-	liter
M	-	molar
MW	-	molecular weight
mg	-	milligram
min.	-	minutes
mL	-	milliliter
MRT	-	mean residence time
mol	-	moles
N	-	normal
Na^+	-	sodium
Na_2EDTA	-	disodium ethylenediaminetetraacetic acid
NaCl	-	sodium chloride
NaOH	-	sodium hydroxide
ng	-	nanogram
nm	-	nanometer
NMR	-	nuclear magnetic resonance
O.D.	-	optical density
OPD	-	ortho-phenylene diamine
q.s.	-	quantity sufficient

RIA	-	radioimmunoassay
RIBA	-	radioimmunobioassay
RNA	-	ribonucleic acid
rpm	-	revolutions per minute
RT	-	room temperature
s.c	-	subcutaneous
S.D.	-	standard deviation
SEM	-	standard error of the mean
$t_{1/2}$	-	terminal elimination half-life
$t_{1/2(\alpha)}$	-	distribution half-life in α-phase
$t_{1/2(ß)}$	-	elimination half-life in ß-phase
t_{max}	-	time of maximum concentration
t.i.d.	-	thrice a day
V_c	-	volume of central compartment
V_d	-	volume of distribution
$V_{d(ss)}$	-	volume of distribution at steady state
®	-	registered trade mark
Å	-	angstrom
μCi	-	microcurie
μg	-	microgram
μL	-	microliter
^3H	-	tritium

Non-Standard Abbreviations

AHF	-	antihemophilic factor
ALB	-	albumin
ALP	-	alkaline phosphatase
ALT	-	alanine aminotransferase
ANOVA	-	analysis of variance
APC	-	activated protein C
APTT	-	activated partial thromboplastin time
AST	-	aspartate aminotransferase
ATU	-	antithrombin unit
BBP	-	blood bank plasma
BUN	-	blood urea nitrogen
$Ca^{+2}TT$	-	calcium thrombin time
CBC	-	complete blood count
CHOL	-	cholesterol
CREAT	-	creatinine
DVT	-	deep venous thrombosis
EPI	-	extrinsic pathway inhibitor
Factor I	-	fibrinogen
Factor II	-	prothrombin
Factor II_a	-	thrombin

Factor III	-	tissue factor, thromboplastin
Factor IV	-	calcium
Factor V	-	proaccelerin
Factor VII	-	proconvertin, stable factor
Factor VIII	-	hemophilic factor A
Factor IX	-	Christmas factor, plasma thromboplastin component, hemophilic factor B
Factor X	-	Stuart-Prower factor
Factor XI	-	plasma thromboplastin antecedent
Factor XII	-	Hageman factor
Factor $XIII_a$	-	fibrin stabilizing factor
FEIBA®	-	factor eight inhibitor bypass activator
GD	-	General Diagnostics
GLU	-	glucose
γ-GT	-	gamma - glutamyl transaminase
GUSTO	-	Global Use of Strategies to Open Occluded Arteries
HCT	-	hematocrit
HELVETICA	-	Hirudin in a European Restenosis Prevention Trial Versus Heparin Treatment in PTCA Patients
HGB	-	hemoglobin
HMW-K	-	high molecular weight kininogen
HIT	-	Hirudin for Improvement of Thrombolysis
HRP	-	horseradish peroxidase

HV1	-	hirudin variant 1
HV2	-	hirudin variant 2
HV3	-	hirudin variant 3
KIU	-	kallikrein inhibitory unit
LACI	-	lipoprotein associated coagulation inhibitor
LDH	-	lactic dehydrogenase
MCH	-	mean cell hemoglobin
MCHC	-	mean cell hemoglobin concentration
MCV	-	mean cell volume
MPV	-	mean platelet volume
NDP	-	normal dog plasma
NIH	-	National Institutes of Health
NRatP	-	normal rat plasma
NRP	-	normal rabbit plasma
OASIS	-	Organization to Assess Strategies for Ischemic Syndromes
OT	-	Organon Teknika
PAF	-	platelet activating factor
PAI-I	-	plasminogen activator inhibitor-I
PEG	-	polyethylene glycol
PHOS	-	phosphorous
pNA	-	para-nitroaniline

PPACK	-	D-phenylalanyl-L-prolyl-L-arginyl chloromethylketone
PTA	-	plasma thromboplastin antecedent
PTC	-	plasma thromboplastin component
PTCA	-	percutaneous transluminal coronary angioplasty
R	-	registered trade mark
RBC	-	red blood cell
RDW	-	red cell distribution width
rH	-	recombinant hirudin
rHV2	-	recombinant hirudin variant 2
rHV2-Lys 47	-	recombinant hirudin variant 2 with lysine residue in position 47.
serpin	-	serine protease inhibitor
SMAC	-	sequential multiple analysis computer
TIMI	-	Thrombolysis In Myocardial Infarction
T PROT	-	total protein
T BIL	-	total bilirubin
TRIG	-	triglycerides
TT	-	thrombin time
tPA	-	tissue plasminogen activator
U	-	units
U ACID	-	uric acid
USP	-	United States Pharmacopoeia

vWF	-	von Willebrand factor
WBC	-	white blood cell
α362	-	human α-thrombin supplied by Dr. Fenton II

CHAPTER I

REVIEW OF LITERATURE

History of Hirudin

Hirudin is the first anticoagulant known to man that was discovered over a hundred years ago, in 1884, in the salivary gland of the medicinal leech, *Hirudo medicinalis* (Haycraft, 1884; Sawyer, 1988). Leeches were commonly used in the once widespread practice of phlebotomy or bloodletting. The art of bloodletting dates back to the Stone Age, when the ancients believed in its ability to purge bad humors from the body (Adams, 1989). An early documentation of the use of leeches for medicinal purposes appears in a painting in an Egyptian tomb around 1500 BC (Sawyer, 1988; Fields, 1991). Leeching has also been documented in the ancient Indian medical encyclopedia known as *Sushruta Samhita*, compiled between 500 BC and AD 200 (Fields, 1991). This was followed by the Romans, Greeks, and most other Europeans. Figure 1 illustrates the use of leeches in ancient medicine, depicting the placement of live leeches on the neck of a patient. Soon, the ugly, bloodsucking leech became the aspirin of the day, as it was being used to treat all kinds of maladies ranging from headaches to insomnia and obesity. Consequently, by the beginning of the 20th century, leech populations in Europe were almost completely depleted, and by 1910, *Hirudo medicinalis* was

Figure 1. Use of leeches in ancient medicine. (Reproduced, by permission, from the lithograph by Louis Boilly, Clements C. Fry Collection, Yale Medical Library, Boilly, Louis Leopold (1761-1845) "Les Sangsues")

declared extinct in Britain (Sawyer, 1988). The overcollection and indiscriminate use of leeches led to the inclusion of this species of the leech in the *Red Data Book of Endangered Species.* The use of leeches in bloodletting was advantageous as their bite was less painful than the wound inflicted by conventional bloodletting instruments. Besides, one can apply the leech to less accessible areas such as tonsils, cervix and hemorrhoids (Adams, 1989). Even today, leeches are being used to restore circulation after microsurgery, plastic surgery and surgical reattachment of amputated extremities (Coniff, 1987; Lent and Dickinson, 1988; Sawyer, 1988; Garcia, 1992; West *et al.*, 1994).

The leech owes this wide popularity to the remarkable array of biologically active constituents of its salivary secretion. The most important among these is **hirudin,** that was discovered from leech extracts by John B. Haycraft in 1884. Haycraft (1884) demonstrated that the active constituent of leech salivary secretion possessed potent anticoagulant properties. This compound was named "hirudin" by Karl Jacoby in 1904 (Jacoby, 1904). This makes hirudin the world's first anticoagulant (Sawyer, 1988), as heparin was isolated from liver in 1916 (McLean, 1916) and bishydroxycoumarin was produced from fermented clover in the late 1930s (Link, 1959). It is interesting to note that Abel *et al.* (1914) had attempted to use hirudin to prevent clotting of blood during research with an "artificial kidney".

The other constituents of the leech salivary secretion include other

proteinase inhibitors such as bdellins which are inhibitors of plasmin and trypsin, eglins which are antagonists of lysosomal polymorphonucleocyte elastase and cathepsin G, pancreatin, chymotrypsin, as well as bacterial subtilin (Seemuller *et al.*, 1986; Fink *et al.*, 1988). The leech salivary secretion also contains other substances that inhibit the amidolytic and kininogenase activities of plasma kallikrein (Baskova *et al.*, 1981), those that have lipase and cholesterol esterase activities (Baskova *et al.*, 1984), and still others that have local anesthetic and hyaluronidase-like activity (Fields, 1991).

Attempts to isolate pure hirudin from leeches were severely hampered due to the shortage of *Hirudo medicinalis* and the lack of sophisticated technical facilities required for such an endeavor. Eventually, Markwardt succeeded in isolating hirudin from medicinal leeches in 1955. Different methods for the isolation and purification of hirudin from leech heads as well as whole leeches, including affinity and ion-exchange chromatography on matrix-bound thrombin have been used (Markwardt and Walsmann, 1967; Bagdy *et al.*, 1973; Walsmann and Markwardt, 1985). Markwardt studied the anticoagulant and antithrombotic properties of hirudin extensively. He showed that hirudin was a highly specific inhibitor of **thrombin**, which is the end point of the first stage of coagulation, thereby preventing the conversion of fibrinogen to fibrin (Markwardt, 1970).

Structural Analysis and Biotechnology of Hirudin

Hirudin is not a single entity, but a group of structurally similar single-chain polypeptides ("hirudins") of a length of 65 or 66 amino acids with an average molecular weight of ≈ 7000 daltons. The complete amino acid sequence of hirudins was determined using automated liquid phase Edman degradation of color-carboxymethylated hirudin and its tryptic peptides (Dodt *et al.*, 1984). The amino acid composition showed an unusually high content of dicarboxylic acids and the absence of arginine, methionine, and tryptophan (Dodt *et al.*, 1984; Seemuller *et al.*, 1986).

Hirudin preparations with different specific thrombin inhibitory activities and different amino-terminal residues have been described (Markwardt and Walsmann, 1967; Bagdy *et al.*, 1973; Graf *et al.*, 1973). Dodt (1984) purified hirudins extracted from whole leeches by gel filtration on Sephadex G-75 and Biogel P 10, as well as anion-exchange chromatography on diethylaminoethyl (DEAE)-cellulose and DEAE-Sephadex A-25. This effort demonstrated the presence of a non-homogeneous population of hirudins with two different amino-terminal residues, valine and isoleucine, in the ratio of 3:1. The hirudin with amino-terminal valine was termed "hirudin" and the hirudin with amino-terminal isoleucine was termed "hirudin PA". Closely related isoforms of hirudin and hirudin PA were designated as hirudin variant 1 (HV1), hirudin variant 2 (HV2), etc., and hirudin PA-1, hirudin PA-2, etc., respectively (Seemuller *et al.*, 1986).

Dodt *et al.* (1986) also determined the structural features of hirudin PA and noted an 82% homology between the acid residues of hirudin and hirudin PA. The more distinct differences are the amino-terminal isoleucine, an additional amino acid (total = 66 amino acids) and the exchange of lysine for glutamine in position 24 in hirudin PA. The tyrosine in position 64 of hirudin PA was found to be sulfated.

Baskova *et al.* (1983) have demonstrated the existence of "pseudo-hirudin" in whole leech extracts with physico-chemical properties similar to hirudin; however, it has virtually no antithrombin activity. "Pseudo-hirudin" could probably be a metabolite of hirudin, as it has been shown to have 20 amino acids less than hirudin and has been found only in the bodies of leeches. Isoleucine is the dominant amino-terminal amino acid residue in the hirudin extracted from leech heads alone, while valine is the dominant amino-terminal residue in "pseudo-hirudin" (Baskova *et al.*, 1983). In whole leeches, Baskova *et al.* (1983) found the ratio of "pseudo-hirudin" to hirudin to be (3-4):1.

Despite the isolation and characterization of the anticoagulant properties of hirudin in the 1950s, active research on hirudin progressed at a slow pace due to the limited availability of natural hirudin. During the past ten years, advances in molecular biological techniques have produced a dramatic improvement, resulting in the production of sufficient quantities of hirudin in the recombinant form (for reviews, see Rieger *et al.*, 1988; Courtney *et al.*, 1989; Johnson *et al.*, 1989; Märki and Wallis, 1990). Harvey *et al.* (1985)

found that there were at least three distinct hirudin transcripts detectable in leech ribonucleic acids (RNAs) that were different in size, site of synthesis, inducibility by starvation and relationship to hirudin activity. This led to the creation of a complementary deoxyribonucleic acid (cDNA) bank isolated from leech heads. The primary structures of three variants of natural hirudin have been determined and designated as hirudin variant 1 (HV1), hirudin variant 2 (HV2) and hirudin variant 3 (HV3). Figure 2 illustrates the amino acid sequence of HV1, HV2 and HV3. It is clear from Figure 2 that the three variants of hirudin exhibit considerable homology ($\approx$ 85%). HV3 is composed of 66 amino acids, while HV1 and HV2 contain 65 amino acid residues. HV1 differs from HV2 and HV3 by 9 and 12 amino acids, respectively. HV2 differs from HV3 by 9 amino acids (Figure 2).

Natural hirudins are mixtures of variants, but **recombinant hirudins (rHs)** are homogeneous preparations (Rydel *et al.*, 1990). Several laboratories have succeeded in the cloning, expression and characterization of rHs from vectors such as *Escherichia coli* (Harvey *et al.*, 1985; Bergmann *et al.*, 1986; Fortkamp *et al.*, 1986; Rieger *et al.*, 1988), *Saccharomyces cerevisiae* (Loison *et al.*, 1988; Reihl-Bellon *et al.*, 1989), and *Bacillus subtilis* (Furutani *et al.*, 1988).

Figure 3 shows a comparison between the structures of natural hirudin and recombinant hirudin. A unique structural feature of natural hirudin is the sulfated tyrosine residue at position 63 (Petersen *et al.*, 1976; Dodt *et al.*, 1984). The covalent structure of hirudin indicates the presence of three

```
      1         10        20        30        40        50        60
HV1   VVYTDCTESG QNLCLCEGSN VCGQGNKCIL GSDGEKNQCV TGEGTPKPQS HNDGDFEEIP EE YLQ

      1         10        20        30        40        50        60
HV2   ITYTDCTESG QNLCLCEGSN VCGKGNKCIL GSNGKGNQCV TGEGTPNPES HNNGDFEEIP EE YLQ

      1         10        20        30        40        50        60
HV3   ITYTDCTESG QNLCLCEGSN VCGKGNKCIL GSQGKDNQCV TGEGTPKPQS HNQGDFEPIP EDAYDE
```

Figure 2. Amino acid sequences of hirudin variants 1, 2 and 3 (HV1, HV2 and HV3).
(Reproduced, by permission, from Märki and Wallis, 1990, "The anticoagulant and antithrombotic properties of hirudin", *Thromb. Haemost.*, 64:p345)

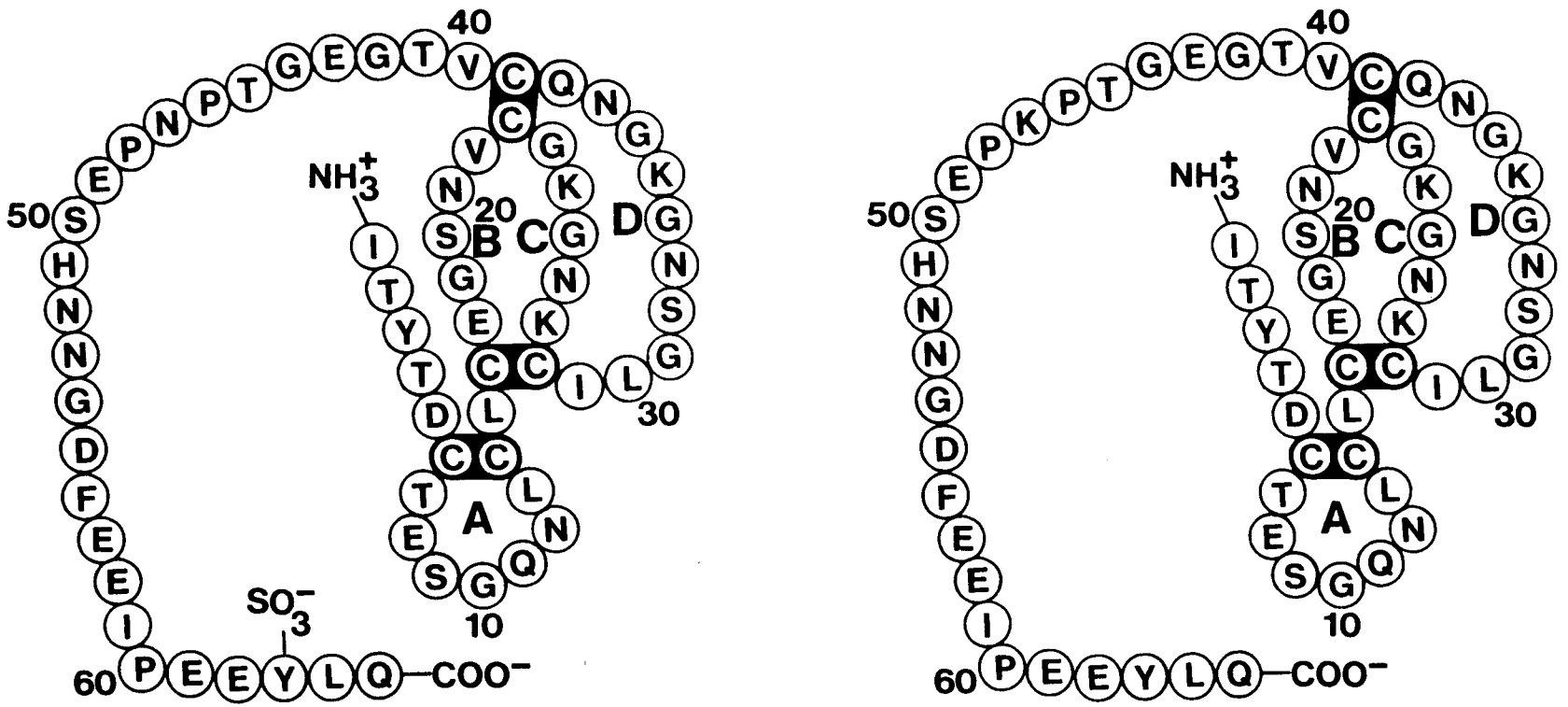


Figure 3. A comparison of structural sequences of natural hirudin and recombinant hirudin variant 2 (rHV2).
(Modified, by permission, from Rydel *et al.*, 1990, "The structure of a complex of recombinant hirudin and human *α*-thrombin", *Science*, 249:p278).

disulfide bridges between Cys6-Cys14, Cys16-Cys28 and Cys22-Cys39 (Dodt *et al.*, 1985). Hirudins appear to have an unusual asymmetry of structural elements consisting of a compact, hydrophobic amino-terminal core region that has alternating polar and non-polar segments, with the three disulfide bonds, and a more extended and highly acidic carboxy-terminal region (Johnson *et al.*, 1989).

All recombinant hirudins produced to date by genetic engineering methods lack the sulfate group on the tyrosine residue at the terminal end, and hence are designated as **"desulfatohirudins"**. The solution structural conformation of desulfatohirudins is similar to that of native hirudins, as determined using nuclear magnetic resonance (NMR) spectroscopy (Haruyama and Wuthrich, 1989). The recombinant form is secreted from the vector with the same unusual asymmetry in structure with a compact amino-terminal region accommodating three disulfide bridges, and an acidic carboxy-terminal (Figure 3).

The amino acid sequence of recombinant form of HV2 (rHV2) is depicted in Figure 4. This variant has been used in all experiments reported in this dissertation. The sequence of rHV2 (and HV2) is also illustrated in Figure 3, indicating the presence of lysine, instead of asparagine, in the recombinant form. Most of the literature on hirudin refers to rHV2 as "rHV2-Lys 47", emphasizing the presence of the lysine group in position 47. The importance of this lysine residue in the mechanism of action of hirudin is discussed later.

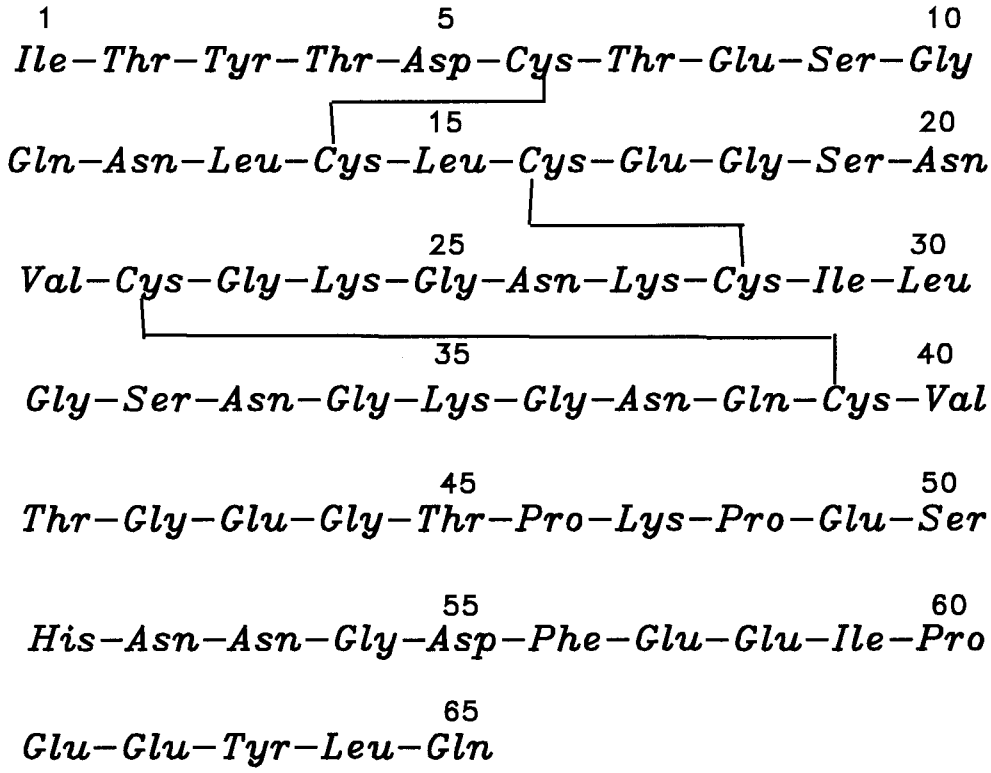


Figure 4. Primary structure of recombinant hirudin variant 2 (rHV2). (Reproduced, by permission, from Tuong *et al.*, 1992, "Characterization of the deamidated forms of recombinant hirudin", *Biochemistry*, 31:p8291.)

The reason for the existence of different isoforms of hirudin is really not known. However, it does suggest the existence of a gene family of thrombin inhibitors (Seemuller *et al.*, 1986). Scharf *et al.* (1989) have identified the primary structures of 10 new hirudins from a native hirudin extract and have supported the hypothesis of a family of hirudins or "isohirudins".

It is important to note that both natural hirudins, as well as their recombinant counterparts may be multimeric in aqueous solution (Johnson *et al.*, 1989), with apparent molecular weights of 51,000 daltons (Braun, 1990). Using circular dichroism spectroscopy, Kunno *et al.* (1988) have demonstrated the cooperative nature of the thrombin-hirudin binding process, suggesting the possibility that the initial binding of thrombin is to the multimeric form of hirudin followed by the dissociation of the multimer to give a tighter binding 1:1 complex (Johnson *et al.*, 1989).

The Coagulation Cascade

Normally, blood flows in a fluid state within a closed vascular system. Upon injury to a blood vessel, two principal mechanisms come into play in order to reduce blood loss (Davie *et al.*, 1991):

i) Activation, adhesion and aggregation of platelets at the site of injury to form a platelet plug that temporarily reduces blood loss, and

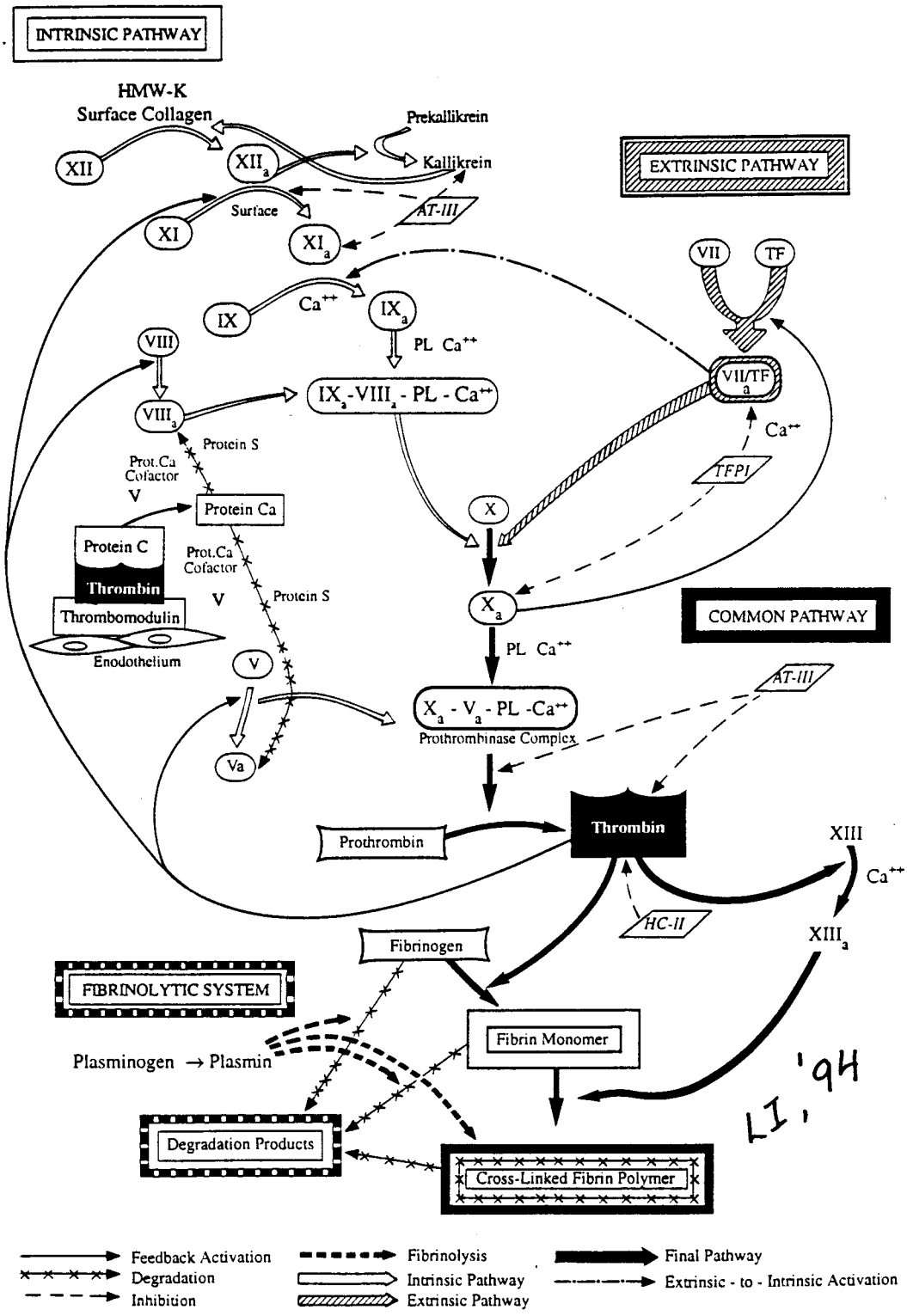
ii) Triggering of a complex series of reactions involving blood clotting factors that lead to fibrin formation and generation of an insoluble fibrin clot, thereby strengthening the platelet plug.

In 1964, two similar, yet independent mechanisms were proposed to explain the process of blood coagulation. These mechanisms came to be known as the 'waterfall' (Davie and Ratnoff, 1964) and 'cascade' (Macfarlane, 1964) theories of blood coagulation. Both theories were based on the observation that inactive precursors (zymogens) of blood clotting factors (denoted with Roman numerals) were sequentially activated to their corresponding active forms (denoted with Roman numerals and suffix 'a') by a process of limited proteolysis. All events were directed toward the conversion of soluble fibrinogen to insoluble fibrin (Roberts and Lozier, 1992).

The various stages in the coagulation cascade are illustrated in Figure 5. In a broad sense, the coagulation scheme can be separated into two pathways, the **intrinsic** and **extrinsic** pathways. The two pathways converge at the level of factor X, leading to the generation of thrombin and fibrin through a single **common** pathway. In the intrinsic pathway of coagulation, all substances required for clotting are normal constituents of blood or are 'intrinsic' to blood. This pathway is triggered by exposure to foreign surfaces such as glass, kaolin, barium carbonate, celite, bentonite and asbestos (Davie and Ratnoff, 1964), or artificial surfaces such as prosthetic heart valve or during blood dialysis (Davie *et al.*, 1991). Four plasma proteins (contact factors) are involved in

Figure 5. The Coagulation Cascade

The Coagulation Cascade



such surface contact, namely, factor XII (Hageman factor), factor XI (plasma thromboplastin antecedent, PTA), prekallikrein (Fletcher factor) and high molecular weight kininogen (HMW-K, Fitzgerald, Flaujeac or Williams factor) (Saito, 1994). It has been proposed that the intrinsic system is activated *in vivo* by contact of factor XII with collagen, chondroitin sulfate, sebum or platelets (Ratnoff, 1974). Upon surface contact, prekallikrein gets activated to kallikrein, which converts factor XII to XII_a in the presence of HMW-K, again, upon surface contact (Saito, 1994). In turn, factor XII_a activates factor XI to factor XI_a (Figure 5). There is recent evidence that factor XI activation can occur independent of factor XII by thrombin in the presence of negatively charged substances such as heparin or dermatan sulfate, or by autoactivation (Naito and Fujikawa, 1991). The generated factor XI_a converts factor IX (Christmas factor, hemophilic factor B, plasma thromboplastin component, PTC) to factor IX_a in the presence of calcium ions (factor IV). Factor IX_a and calcium ions are assembled in close proximity to factor X (Stuart-Prower factor) on platelet surface, in the presence of activated factor VIII ($VIII_a$), thereby promoting the conversion of factor X to X_a.

The extrinsic pathway of coagulation requires the presence of tissue factor (TF, factor III, thromboplastin) that is located in tissue adventitia and comes in contact with blood only after vascular injury. Hence, this pathway is known as the **tissue factor pathway** of coagulation (Broze, 1994). Tissue factor is an integral membrane glycoprotein that is tightly associated with

phospholipids. During vascular injury, tissue factor is released to form a 1:1 stoichiometric complex with factor VII (proconvertin, stable factor) in the presence of calcium ions, leading to the activation of factor VII to factor VII_a (Nemerson and Repke, 1985). It has also been proposed that factor VII, in complex with tissue factor, is activated by factor X_a circulating in trace amounts in plasma (Roberts and Lozier, 1992). Factor VII_a also binds to tissue factor with equal affinity as inactivated factor VII. The TF/VII_a complex (convertin) activates substrates factor X to X_a as well as factor IX to IX_a, presumably by tissue factor's cofactor effect of inducing a conformation change in factor VII (Broze, 1994). The activation of factor IX by TF/VII_a complex is a demonstration of extrinsic-to-intrinsic activation in the coagulation cascade (Roberts and Lozier, 1992).

The intrinsic and extrinsic pathways converge at the step of activation of factor X to X_a. The generated X_a forms a 1:1 complex with activated factor V (V_a), in the presence of calcium ions and phospholipids, known as **prothrombinase complex**. This complex activates prothrombin (factor II) to thrombin (factor II_a) (Davie *et al.*, 1991). It is important to note that factors II, VII, IX, X, as well as protein C and protein S (see below) require Vitamin K for the formation of their active forms (Roberts and Lozier, 1992).

The generated thrombin transforms fibrinogen (factor I) to fibrin monomers. The individual monomeric fibrin units are polymerized into tough, elastic fibers, resulting in clot formation. The stabilization of the clot occurs

when fibrin monomers, in the presence of calcium ions, are linked side-to-side by factor $XIII_a$ (fibrinase, fibrin stabilizing factor). Factor $XIII_a$ combines the beta-carboxyl group of asparagine in one fibrin monomer to an amino-terminal glycine residue in an adjacent monomer (Ratnoff, 1965). The fibrin clot is dissolved by fibrinolytic enzymes such as plasmin as part of a process of wound healing.

Feedback activation by thrombin. Once formed, thrombin plays a major role in sustaining coagulation through feedback activation of coagulation factors. Thrombin activates factor V (proaccelerin) to V_a, factor VIII (Hemophilic factor A, antihemophilic factor, AHF) to $VIII_a$ and factor XIII to $XIII_a$. As mentioned above, there is recent evidence that thrombin also activates factor XI to XI_a (Naito and Fujikawa, 1991).

Other feedback mechanisms. The coagulation cascade exhibits certain other feedback mechanisms such as the activation of prekallikrein by factor XII_a (Cochrane *et al.*, 1973) as well as the activation of factor VII by factor X_a (Roberts and Tozier, 1992).

Regulation of the coagulation cascade. The endothelium expresses a transmembrane protein known as thrombomodulin which serves as a receptor to thrombin. Thrombin, bound to thrombomodulin, is changed from a procoagulant to an anticoagulant, as it activates protein C to protein C_a

(activated protein C, APC) (Davie *et al.*, 1991). Protein C_a is a key component of a natural anticoagulant pathway (Dahlback and Stenflo, 1994) that catalyzes the proteolytic degradation of factors V_a and $VIII_a$ (Figure 5). Protein C requires protein S for this activity (Walker, 1981). As coagulation proceeds and more thrombin is formed at higher concentrations, thrombin inhibits cofactors V_a and $VIII_a$ by this negative feedback mechanism.

Recently, there has been another new evidence for a balance between procoagulant and anticoagulant properties of clotting factors (Dahlback and Hildebrand, 1994). Factor V has been shown to be a cofactor to protein C_a (APC cofactor, protein C_a cofactor). A defect in anticoagulant response to protein C_a (APC resistance) was observed in familial thrombophilia patients. Plasma from APC-resistant patients has been shown to have normal factor V procoagulant activity and abnormal factor V anticoagulant activity, indicating a possible mutation(s) in factor V gene affecting factor V anticoagulant function, selectively (Dahlback and Hildebrand, 1994). This new finding will have a substantial impact on our current knowledge of blood coagulation and its regulation (Bauer, 1994).

Antithrombin III (AT-III) is a protein that inhibits not only thrombin but also other serine proteases such as factors X_a, IX_a, XI_a, and XII_a, and protein C_a. The anti-thrombin and anti-X_a activity of AT-III is markedly enhanced by heparin (Bick, 1992a, 1992b). Another endogenous thrombin inhibitor is heparin cofactor II (HC-II) that also inhibits chymotrypsin (Bick, 1992a, 1992b).

Other plasma serine protease inhibitors (serpins) that play a significant role in the regulation of coagulation include α_2-macroglobulin, protein C_a inhibitor, C1-esterase inhibitor and α_1-antitrypsin (Davie *et al.*, 1991).

Most blood coagulation reactions occur on a phospholipid surface. Activation of platelets by small amounts of thrombin causes internal phospholipids to be flipped out to the outside of platelets. Platelets are rich in phospholipids such as phosphatidylserine and phosphatidylcholine (Roberts and Lozier, 1992). The endothelium also contributes significantly to hemostasis by expression of thrombomodulin, tissue factor pathway inhibitor (TFPI), TF (induced by endotoxins, tumor necrosis factor), platelet activating factor (PAF), von Willebrand factor (vWF), tissue plasminogen activator (tPA) , plasminogen activator inhibitor-I (PAI-I), cytokines, as well as by providing binding sites to coagulation factors V_a, X_a and IX_a (Pearson, 1994).

Revised hypothesis of blood coagulation. The discovery of a protein known as tissue factor pathway inhibitor (TFPI) led to a revised hypothesis of blood coagulation (Broze, 1992). TFPI, produced by endothelial cells, is also known as anticonvertin, antithromboplastin, factor X_a-dependent factor VII_a/TF inhibitor, the tissue factor inhibitor, the extrinsic pathway inhibitor (EPI) or the lipoprotein-associated coagulation inhibitor (LACI). It was found that patients with defects in either the intrinsic pathway (factor VIII and IX deficiency) or extrinsic pathway (factor VII deficiency) exhibit bleeding whereas people

lacking one of the 'contact' factors do not bleed excessively (Broze, 1992, 1994). This observation demonstrated that the earlier 'waterfall' and 'cascade' hypotheses did not describe *in vivo* hemostatic mechanisms, accurately. According to the revised theory, factor VII or VII_a bind to TF exposed due to blood vessel damage. The VII_a/TF complex activates factor X to X_a and factor IX to IX_a. Once factor X_a is formed, TFPI inhibits the VII_a/TF complex in a X_a-dependent manner that also requires calcium ions (Figure 5) (Broze *et al.*, 1988). Further production of factor X_a and IX_a by the VII_a/TF complex is prevented. Additional factor X_a is generated through the alternative intrinsic pathway via factor IX_a and $VIII_a$.

The revised model explains the requirement of both intrinsic and extrinsic factors *in vivo* for normal hemostasis (Broze, 1992). This model integrates all the factors into a single pathway triggered by the factor VII_a/TF complex eliminating the requirement for contact factors. The initially formed factor X_a generates sufficient thrombin to induce platelet aggregation and factor V and factor VIII activation. However, for sustained hemostasis, additional factor X_a is required to be produced through factor $VIII_a$ and factor IX_a (Broze, 1992).

Mechanism of Antithrombin Activity of Hirudin: The Hirudin-Thrombin Interaction

Hirudin is a highly specific inhibitor of thrombin, the serine protease glycoprotein that plays a key regulatory role in hemostasis and thrombosis. Hirudin forms a 1:1 stoichiometric complex with α-thrombin with a dissociation

constant, $K_i = 50$ pM/L, indicating an exceedingly strong and high-affinity complex (Markwardt and Walsmann, 1958). Hirudin activity is expressed as a function of thrombin activity which is expressed in National Institutes of Health (NIH) units. One antithrombin unit (ATU) of hirudin corresponds to the amount of hirudin which neutralizes one NIH unit of thrombin (Markwardt, 1970).

The scheme of interaction between hirudin and thrombin, as reported by Markwardt (1991b), is illustrated in Figure 6. The structure of hirudin complements the structure of thrombin. Winant *et al.* (1991) have shown that specific segments within both the amino- and the carboxy-terminal residues of hirudin interact with thrombin. The secondary and tertiary structural conformations of hirudin are important for its antithrombin activity as the activity is lost during oxidation and reduction of the disulfide bridges, and proteolytic degradation of the peptide (Chang, 1983; Markwardt, 1988).

Hirudin inactivates thrombin by a two-step process. In the first step, the C-terminal region of hirudin binds to the anion-binding (fibrinogen binding) exosite region of thrombin (Figure 6). This results in a slight conformational change in the enzyme (Johnson, 1994). The second step involves the binding of the hydrophobic core of the amino-terminal region of hirudin to the active site (catalytic) domain of thrombin. Upon hirudin binding, a loop of ten amino acid residues from the catalytic site of thrombin is displaced, resulting in loss of thrombin catalytic activity (Johnson, 1994).

Extensive structural studies have been performed to study the mechanisms involved in the hirudin-thrombin interaction (Rydel *et al.*, 1990; Rydel *et al.*, 1991; Johnson *et al.*, 1989; Johnson, 1994; Stone and Maraganore, 1994). The crystallographic structure of a complex of rH (rHV2) and human α-thrombin was studied by Rydel *et al.* (1990, 1991). It was found that the primary basic specificity pocket ("arginine side chain pocket") of thrombin is not occupied by hirudin, in contrast to that shown in Figure 6. The lysine residue in position 47 was not found to occupy the basic specificity pocket of thrombin. Instead, the ε-amino group in lys-47 was found to help maintain the conformation of the amino-terminal tripeptide to penetrate and form a hydrogen bond with serine-195 of the thrombin active site region (Rydel *et al.*, 1991). A conformational change in thrombin has been observed after its interaction with hirudin, using circular dichroism (Kunno *et al.*, 1988). In all, 27 of the 65 amino acids of rHV2 were found to have contacts that were less than 4.0 Å with thrombin, with 10 ion pairs and 23 hydrogen bonds (Rydel *et al.*, 1990). These abundant interactions may explain the specificity and high affinity of hirudin for thrombin.

Alterations in the amino-terminal region of hirudin can destabilize the interaction of other regions with thrombin. Nitration of tyrosine-3 increases the polarity and hence, may destabilize hirudin's interaction with the apolar binding site of thrombin. Replacing the tyrosine-3 residue with tryptophan or

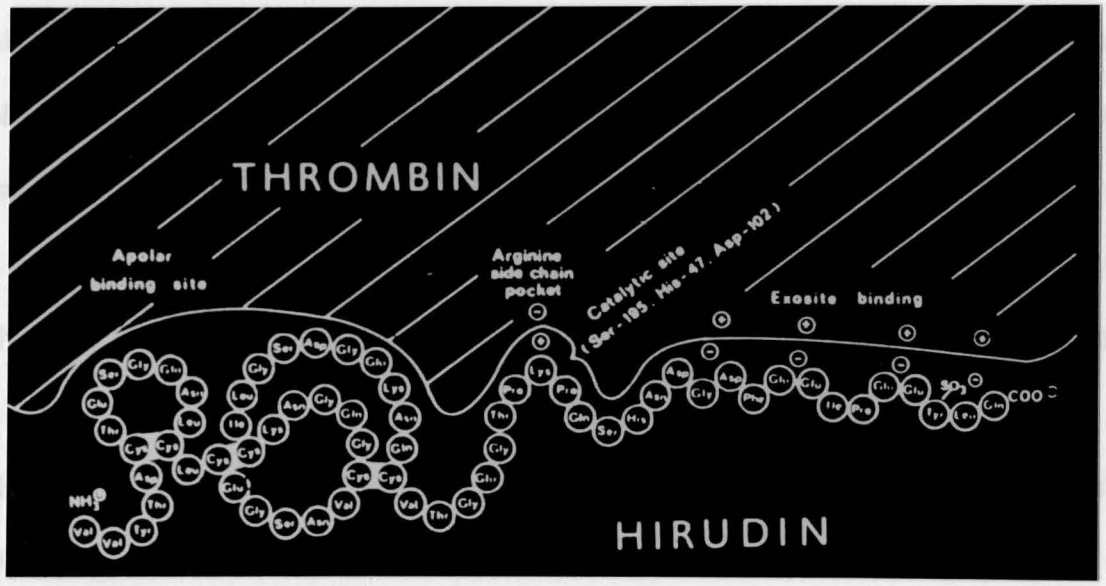


Figure 6. Scheme of hirudin-thrombin interaction.
(Reproduced, by permission, from Markwardt, 1991b, "Past, present and future of hirudin", 21(suppl. 1):p13.)

phenylalanine increases the affinity of hirudin for thrombin (3-6 fold), whereas substituting with threonine resulted in 450-fold increase in the K_i (Lazar *et al.*, 1991). Replacement of lys-47 with glutamic acid in rH results in a 200-fold loss in affinity (Dodt *et al.*, 1988), indicating that this lysine group contributes to the overall affinity of hirudin for thrombin.

The interaction between recombinant form of hirudin and thrombin is considerably strong, despite a 10-fold increase in K_i, as the K_i for the natural hirudin-thrombin complex is in the picomolar range (Markwardt, 1991b). The significance of the sulfation of tyrosine-63 in native hirudin is not clear. Nitration or iodination of tyrosine-63 of rH restores hirudin-thrombin affinity to levels similar to or exceeding that of native hirudin (Winant *et al.*, 1991).

Cleavage of arginine residues within the anion-binding exosite of thrombin results in the formation of β-thrombin with a loss of fibrinogen clotting activity. In humans, β-thrombin is subsequently converted to γ-thrombin, for which hirudin has 2-3 fold lesser affinity than for α-thrombin (Fenton II, 1989; Fenton II *et al.*, 1991).

Thrombin is the activated form of prothrombin and it modulates a variety of functions including activation of plasma proteins such as fibrinogen, coagulation factors such as factors V, VIII, and XIII and protein C, stimulation of blood cells such as leukocytes and platelets, and the stimulation of endothelial cells and smooth muscles. Thrombin also exhibits receptor-mediated hormone-like actions including monocyte and neutrophil chemotaxis.

Most of the actions of thrombin are controlled by physiological inhibitors such as antithrombin III, heparin cofactor II and α_2-macroglobulin (Fenton II, 1989).

The anticoagulant effect of hirudin is unique as it is highly specific for α-thrombin without any significant effect on other serine proteases. In addition, hirudin is a direct antagonist of thrombin, without requiring the presence of cofactors like antithrombin III and heparin cofactor II. Once hirudin binds to thrombin, all proteolytic functions of thrombin are blocked. Hence, not only does hirudin block the formation of the fibrin clot by inhibiting the conversion of fibrinogen to fibrin, but hirudin also blocks thrombin-catalyzed activation reactions like feedback activation of factors V, VIII, and XIII (Figure 5), thrombin-induced platelet, endothelial and smooth muscle cell reactions. (Markwardt, 1988; Markwardt, 1991b). Hirudin also prevents the activation of the endogenous anticoagulant, protein C, to protein C_a by thrombomodulin-thrombin complex (Figure 5). However, it has been reported that this may not attenuate the anticoagulant effects of hirudin as protein C inactivates only the activated forms of factors V and VIII, which are absent in the presence of hirudin (Markwardt, 1991b).

The ultimate physiological mechanism of the anticoagulant action of hirudin has been proposed to be the inhibition of thrombin generation (Fenton II *et al.*, 1991). The reason for this is the blockade, by hirudin, of thrombin-mediated feedback activation to form more thrombin, via the activation of factor V to V_a and the formation of the prothrombinase complex (Figure 5).

Laboratory Assays for Hirudin

Table 1 lists the different assay methods that are applicable for monitoring hirudin activity and concentration. Most earlier assay methods for hirudin were based on global coagulation tests, such as thrombin time (TT), activated partial thromboplastin time (APTT), and more recently, the modified TT, known as calcium thrombin time (Ca^{+2}TT). Also available is the amidolytic anti-IIa assay, based on the cleavage of p-nitroaniline from a thrombin-specific substrate, in the presence of hirudin. The above assay methods are based on indirect pharmacodynamic measurements.

A major breakthrough in the development of laboratory methods to monitor hirudin was the generation of hirudin-specific antisera in sheep, despite the fact that hirudin was a poor immunogen (Spinner *et al.*, 1986). Immunological methods enable the direct measurement of rH concentration in biological fluids. Radioimmunoassays (RIA) (Bichler *et al.*, 1988) and enzyme-linked immunosorbent assays (ELISA) (Spinner *et al.*, 1986; Spinner *et al.*, 1988; Amiral *et al.*, 1991; Berscheid *et al.*, 1992; Mille *et al.*, 1994; Iyer *et al.*, 1995) are currently being developed for rH measurement.

The functional assays have an advantage of being able to evaluate the actual functionality of rH as an anticoagulant. This is particularly important in the clinical setting as the anticoagulant effect of rH is the only useful

TABLE 1

LABORATORY ASSAYS FOR THE DETERMINATION OF RECOMBINANT HIRUDIN

Functional Assays	Non-functional Assays
Whole Blood Clotting Time	Radioimmunoassay (RIA)
Bleeding Time	Enzyme-Linked Immunosorbent Assay (ELISA)
Prothrombin Time (PT)	High Performance Liquid Chromatography (HPLC)
Tissue Factor Clotting Time (TFCT)	Circular Dichroism (CD) Spectroscopy
Activated Partial Thromboplastin Time (APTT)	Mass Spectrometry
Thrombin Time (TT)	Photometric Assay
Calcium Thrombin Time (Ca^{+2}TT)	X-ray Crystallography
Heptest	
Anti-IIa (amidolytic) Assay	
Thrombin Generation Assay	
Ecarin Clotting Time (ECT)	

(Modified, by permission, from Fareed *et al.*, 1991b, "An objective perspective on recombinant hirudin: a new anticoagulant and antithrombotic agent", *Blood Coag. Fibrinol.*, 2:p138.)

parameter in this situation (Fareed *et al.*, 1991b; Walenga *et al.*, 1991a). On the other hand, the immunoassays, if designed to be specific for the parent drug without cross-reactivity to other peptides or rH metabolites, offer a direct measurement of absolute concentration of rHs in biological fluids. This would aid in the study of pharmacokinetic characterization of rHs.

High performance liquid chromatography (HPLC) is another method

(Chang, 1991; Groetsch *et al.*, 1991) that is being developed to assay rH. This method could prove to be more useful in the study of the integrity and stability of hirudin in solution and in biological fluids. However, unlike the ELISA or comparable methods, HPLC lacks the ability to discriminate between active and inactive forms of hirudin. More recently, newer methods are being developed to study the structural features of hirudin and binding characteristics of hirudin with thrombin, such as mass spectrometry (Van Dorsselaer *et al.*, 1989), photometric assay (Spannagl *et al.*, 1991), crystallography (Rydel *et al.*, 1990, 1991) and circular dichroism spectroscopy (Kunno *et al.*, 1988).

Pharmacokinetics of Hirudin

Like any therapeutic agent, the efficacy of an antithrombotic agent depends on the maintenance of adequate levels of the drug in the blood, which, in turn, is likely the site of action of antithrombotic drugs. At the same time, the drug must not exceed therapeutic concentrations in the plasma as, ultimately, too high concentrations in the plasma may lead to toxic effects. To achieve these goals, a complete working knowledge of the pharmacokinetic behavior of the drug is essential. This is important in the selection of a dose, a dosage form, frequency of administration, and route of administration during its clinical use. The relationship between pharmacokinetics and pharmacodynamics of hirudins is not fully understood despite the fact that many research groups have published information regarding the disposition of

this drug after intravenous (i.v. bolus and i.v. infusion) and subcutaneous (s.c.) administration. Tables 20 to 23 in Appendix I highlight the features of some of the pharmacokinetic studies performed on hirudin.

With regard to absorption, hirudin, being a polypeptide, is not expected to be absorbed significantly after oral administration. Markwardt *et al.* (1988a) found that there was no marked absorption after rectal administration of rH (1 mg/kg) in rats, despite the hydrophobic nature of the hirudin molecule.. There have been conflicting reports in the literature about the bioavailability of hirudins after s.c. administration. Markwardt *et al.* (1984) reported a 36% bioavailability of natural hirudin in humans, while the same group of workers reported almost complete absorption of rH in humans (Markwardt *et al.*, 1988b) and in rats (1988a). Bichler *et al.* (1988) have reported a subcutaneous bioavailability of 85% in humans with natural hirudin.

Markwardt *et al.* (1984) studied the disposition of natural hirudin in rats, rabbits, and dogs (1982) and in humans. After i.v. administration, natural hirudin appears to be rapidly distributed into the tissue compartment, with a $t_{1/2(\alpha)}$ of about 10 minutes, followed by a slower elimination phase ($t_{1/2(ß)}$) of about 60 minutes. The plasma concentrations, after being determined by chromogenic thrombin substrate assay were fit to a biexponential equation, $C_p = Ae^{-\alpha t} + Be^{-ßt}$, where C_p is the concentration at time t, A and B are constants and α and ß are the distribution and elimination rate constants, respectively. Similarly, other investigators have described hirudin

pharmacokinetics using an open two compartment model after i.v. administration (Bichler *et al.*, 1988; Nowak *et al.*, 1988; Richter *et al.*, 1988; Meyer *et al.*, 1990). However, one group of researchers has not excluded the existence of a third compartment (Meyer *et al.*, 1990), predicting a γ phase of elimination.

The volume of distribution of about 0.2 L/kg in humans indicates that hirudin distributes in the extracellular fluid compartment (Bichler *et al.*, 1988). The distribution characteristics of rH were studied by Markwardt *et al.* (1988b) five hours after i.v. administration of 1 mg ^{125}I-rH/kg in rats, when a relatively uniform pattern of distribution was seen in fat, brain, heart, liver, lungs, spleen, skeletal muscle and pancreas, with a certain amount of accumulation in the kidneys. Markwardt *et al.* (1990) prepared a conjugate of recombinant hirudin with dextran. Dextran-hirudin exhibited a lower volume of distribution than rH, thereby allowing for the drug to be restricted to the effector site, i.e., blood.

Both natural as well as recombinant hirudin have been found to be eliminated unchanged, predominantly via the kidneys. Up to 70 to 90% recovery of hirudin was found in the urine after i.v. administration in different species, including humans (Markwardt *et al.*, 1982; Richter *et al.*, 1988; Markwardt *et al.*, 1988a). Nowak *et al.* (1988) have shown that plasma rH levels remain unchanged for up to 120 minutes after i.v. and s.c. administration in nephrectomized dogs, thereby substantiating the evidence for the role of kidneys in the clearance of hirudin. However, urinary excretion of

hirudin has been found to be lower (≈ 15 %) in rats (Richter *et al.*, 1988; Markwardt *et al.*, 1988a). Urinary recovery of hirudin after s.c. administration is lower (≈ 30-40%) than that obtained after i.v. administration. It appears that the renal clearance of rH is more pronounced (25% increase) than natural hirudin. The lack of the sulfate group in recombinant hirudin in position 63 has been speculated to be the cause of this finding (Nowak *et al.*, 1988; Markwardt *et al.*, 1988a).

The elucidation of the pharmacokinetic characteristics of hirudins has been hampered due to the lack of availability of a specific assay methodology to determine exact levels of hirudin in the central compartment. Most pharmacokinetic studies employed the use of thrombin clotting tests or the chromogenic thrombin substrate assay method. Bichler *et al.* (1988) were the first to use a direct method (radioimmunobioassay/RIBA) to measure plasma and urine concentrations of this drug. Newer studies, including the experiments reported in this dissertation, are being developed using an ELISA method to measure plasma concentrations of rH. It remains to be established whether different isoforms of natural hirudin and rH would exhibit different absorption, distribution, metabolism and excretion patterns. Markwardt *et al.* (1989) have reported the pharmacokinetic characteristics of rHV1 (Appendix I). More studies are required to address the influence of different structural features in the disposition of hirudins after different modes of administration. Furthermore, it is essential to study the disposition of rHs in a target patient

population.

Comparison of Hirudin with Heparin

The major differences between rH and heparin in terms of their chemical composition, mechanism of action, and other factors, are listed in Table 2. The most significant advantage that hirudins offer when compared to the conventional anticoagulant, heparin, is that unlike heparin, the anticoagulant action of hirudin is not mediated by circulating AT-III or HC-II. When the anticoagulant properties of rH and heparin were evaluated in AT-III depleted human plasma, HC-II depleted human plasma, and purified human fibrinogen as compared to normal human plasma, heparin exhibited only a 28%, 74%, and 0% recoverable activity, respectively (Walenga *et al.*, 1990). On the other hand, rH exhibited a 100% recoverable activity in all three systems. Hence, rH can be used conveniently in AT-III and HC-II deficient patients. Moreover, hirudin is resistant to platelet factor 4 which neutralizes the anticoagulant activity of heparin. The anticoagulant effect of hirudin is more uniform than that of heparin as the activity does not appear to be mediated by physiological factors (Doutremepuich *et al.*, 1991).

Doutremepuich *et al.* (1989) compared the antithrombotic activity of rH with that of heparin in an experimental model of stasis-induced venous thrombosis in rats. Hirudin, at a dose of 12.5 μg/kg produced a comparable antithrombotic effect, measured in terms of reduction in thrombus weight, to

TABLE 2

COMPARISON OF RECOMBINANT HIRUDIN WITH HEPARIN

Recombinant Hirudin	Heparin
Monocomponent protein with single target (thrombin)	Multicomponent sulfated polysaccharide with multiple sites of action (thrombin, X_a, XII_a)
Not dependent on cofactors	Cofactor dependent (AT-III and HC-II)
Known activity against clot-bound thrombin	Limited activity against clot-bound thrombin
Activity not affected by endogenous factors such as platelet factor 4, factor VIII and other proteins	Marked modulation by endogenous factors, such as platelet factor 4
No known interaction with endothelium, other than indirect blockade of protein C_a and thrombomodulin-bound thrombin	Significant interactions with endothelium
Relatively inert, not altered by metabolic processes	Transformed by several enzymes reducing anticoagulant action
May not require neutralization No major allergic reactions	Bleeding complications, requires neutralization Known allergic reactions (thrombocytopenia)
No known profibrinolytic effects	Known profibrinolytic effects
Does not release TFPI	Known to release TFPI
Dose adjustments required in patients with compromised renal failure	

400 μg/kg of unfractionated heparin. Porta *et al.* (1990) found that the *in vivo* antithrombotic activity of hirudin matches that of heparin, measured in terms of ED_{50} values (ED_{50} = 0.205-heparin and 0.290-rH) in rats. However, the *in vitro* anticoagulant properties of rH were found to be greater than that of heparin using clot-based functional assays such as activated partial thromboplastin time (APTT) and thrombin time (TT).

The observed side effects of heparin such as bleeding, thrombocytopenia and alopecia are not observed with rH. The antithrombotic and hemorrhagic effects of three variants of rH were compared with that of heparin in rats (Doutremepuich *et al.*, 1991). The reduction in thrombus size produced by the rHs varied, depending on the rH used. Higher doses of heparin (400 μg/kg), but not of the rHs, produced increased bleeding time in a rat tail bleeding model. Walenga *et al.* (1990) found that the hemorrhagic effect of rH was much weaker than heparin at equivalent gravimetric doses. It was found that approximately 4-fold higher gravimetric doses of rH would be needed to obtain the same bleeding effect as heparin (Walenga *et al.*, 1990). However, there have been recent reports of hirudin exhibiting severe hemorrhagic effects in a target patient population, as discussed later.

Heparin has been shown to release TFPI (Valentin *et al.*, 1992; Zitoun *et al.*, 1994), whereas there is no evidence of TFPI being released by hirudin (Fareed, 1994). Another important aspect in the differences between heparin and hirudin is the pharmacokinetic behavior exhibited by these agents. Heparin

exhibits non-linear pharmacokinetics that is dose and time dependent (Bjornsson and Levy, 1979a; Bjornsson and Levy, 1979b), requiring close monitoring of the patient. On the other hand, the results from Meyer *et al.* (1991), as well as some of the results reported in this dissertation, indicate that hirudin may exhibit linear pharmacokinetics. This fact can be utilized for a more efficient selection of a dose and dosing schedule for hirudin than for heparin.

A major limitation of heparin is its ineffectiveness to inhibit fibrin-bound thrombin (Weitz *et al.*, 1990; Johnson, 1994). The fibrin that is formed after cleavage of fibrinogen by thrombin, binds to thrombin at a site distinct from the catalytic site. Fibrin-bound thrombin is enzymatically active and may cleave fibrinogen in the presence of AT-III or HC-II, that would inhibit free thrombin (Johnson, 1994). On the other hand, hirudin was found to be effective against clot-bound thrombin, as neither hirudin nor the carboxy-terminal fragment of hirudin, displaced thrombin from fibrin. It may be possible that thrombin binds fibrin in a manner permitting the fibrinogen-thrombin interaction, at another distinct exosite region (Johnson, 1994).

Neutralization of Recombinant Hirudin

rH has an extremely short half-life of about 30 to 45 minutes. Hence, it may not require an antagonist to neutralize its effects. Fareed *et al.* (1991a, 1991b) have proposed that in normal individuals without any hemostatic

deficit, rH at therapeutic levels should not produce any effect on bleeding. However, considering the crucial indications it is proposed to be used for, an antagonist to neutralize its anticoagulant action may be necessary. A hirudin antidote may be essential in case of accidental overdose or in case of patients with renal disease. Potential candidates that are currently being investigated as possible hirudin antagonists include di-isopropylphosphoryl-thrombin (DIP-thrombin; Bruggener *et al.*, 1989), recombinant factor VIIa (Fareed *et al.*, 1991a), 1-desamino-8-arginine vasopressin (DDAVP; Ibbotson *et al.*, 1991; Butler *et al.*, 1993), factor VIII (Butler *et al.*, 1993), batroxobin (Markwardt *et al.*, 1992), ecarin-induced formation of meizothrombin (Nowak and Bucha, 1994) and activated prothrombin complexes such as factor VIII inhibitor bypass activator (FEIBA®; Fareed *et al.*, 1991a; Stuever *et al.*, 1995) and autoplex® (Diehl *et al.*, 1994). Further studies are needed to evaluate these antidotes in a more clinical situation.

Potential Clinical Use of Recombinant Hirudin (rH)

The importance of antithrombotic drugs in clinical use can be realized from the fact that "thrombosis is a major cause of death and disability resulting from the occlusion of diseased arteries and veins which leads to myocardial infarction, stroke, peripheral ischemia, and pulmonary embolism" (Fuster and Verstraete, 1992).

In terms of its clinical antithrombotic and anticoagulant activity, rH offers

some obvious advantages over the conventional anticoagulant, heparin, as described earlier in this review. However, despite the fact that rH is a stronger antithrombotic agent than heparin, the thrombin generation pathways in the coagulation cascade appear to be inhibited only under certain conditions. rH has been shown to be not as effective as heparin in the generation of thrombin (Kaiser *et al.*, 1992). This could mean that a higher dose of rH may be required as compared to heparin for effective antithrombotic activity, as only one target site can be inhibited by rH (Fareed *et al.*, 1989; Fareed *et al.*, 1991a).

Vogel *et al.* (1988) showed that natural hirudin produced therapeutic effects in chronic disseminated intravascular coagulation (DIC) patients at a dose of 1,000 ATU/kg (s.c., three times daily). rHs have also been proposed to be useful in prevention of restenosis after coronary angioplasty. Rabbits receiving rH (1 mg/kg i.v. bolus, 1 mg/kg/hour i.v. infusion for 1 hour) at the time of experimental balloon angioplasty exhibited significantly less restenosis than rabbits treated with heparin (150 U/kg, i.v. bolus), using angiography and quantitative histopathology (Sarembock *et al.*, 1991). rH totally inhibited arterial thrombosis and limited platelet aggregation to a single layer in a pig model of balloon angioplasty with deep arterial injury (Chesebro *et al.*, 1991a, 1991b). However, many investigators proposed a very cautious use of rH in the treatment of DIC and the prevention of reocclusion after percutaneous transluminal coronary angioplasty (PTCA) at a discussion forum on rH, held at

the Loyola University Chicago, Maywood, IL (Walenga *et al.*, 1991c).

rH may be used in the prophylaxis of deep venous thrombosis in high thrombotic risk patients (Raake *et al.*, 1991). rH is being investigated as an alternative to heparin to provide anticoagulation in a canine cardiopulmonary bypass model (Walenga *et al.*, 1991b). It may be used as an adjunct drug to enhance the antithrombotic properties of other anticoagulant and thrombolytic agents which may be useful in the prevention of reocclusion during thrombolysis (Fareed *et al.*, 1991a).

The use of rH in microvascular surgery is also being investigated. Hubbard *et al.* (1991) showed that rH (125 μg/kg i.v. bolus, 75 μg/kg/hour i.v. infusion) produced a similar patency rate as heparin in a rabbit anastomosis model. A promising biomedical application of rH is in the coating of artificial surfaces to provide a non-thrombogenic surface (Fareed *et al.*, 1991a). rH may be used to provide a non-thrombogenic coating surface in surgical tubing, catheters, membranes, extracorporeal oxygenators, blood collection apparatus, hemodialysis units, Jarvik® (artificial) hearts and other cardiac devices. Unlike heparin, which is currently being studied for use in such devices, rH may not activate platelets and does not require an endogenous cofactor to exert its anticoagulant activity. Bucha *et al.* (1990) studied the use of rH as an anticoagulant in hemodialysis in nephrectomized dogs. In contrast to heparin, rH did not induce thrombocytopenia or bleeding in such dogs.

rH has been proposed to be useful in diagnostic tests. Hirudin may be

used as a tool for the control of catalytic and non-enzymatic activities of thrombin (Stocker, 1991). As rH is highly specific in its action against thrombin, it may be used, in conjunction with chromogenic substrates, to discriminate between actions mediated by thrombin, its precursors, cofactors and effectors and actions of other enzyme systems. rH may be a very useful anticoagulant for experimental biomedical research applications. Unlike heparin and calcium chelating agents such as citrate and EDTA (ethylenediamine tetraacetic acid), rH does not produce any chelation with trace metals, in particular, magnesium and calcium, which are important in biological functions of cells and intact preparations. Thus, rH may be an anticoagulant of choice for experimental pharmacological preparations such as tissue culture and isolated tissue preparations.

Clinical trials with rH. A major concern over the use of antithrombotic drugs is the risk of bleeding, particularly when used in conjunction with thrombolytic agents, invasive procedures and patient's own predisposing factors (Johnson, 1994). In particular, additional precautions may be required during use of specific thrombin inhibitors such as rH, due to the strong antithrombotic potency of such drugs. rH is currently undergoing clinical trials for indications such as myocardial infarction, unstable angina, and PTCA (Lefkovits and Topol, 1994).

The phase I studies performed on normal, human volunteers indicated

that rHs were well tolerated, without any adverse effects in therapeutic doses (Markwardt *et al.*, 1984; Bichler *et al.*, 1988; Verstraete *et al.*, 1993; Hoet *et al.*, 1994). Bleeding time was not prolonged even after use of rH at a dose of 0.5 mg/kg (Verstraete *et al.*, 1993). The results from phase II clinical trials using rH were encouraging, as angiographic improvement was evident in 116 patients with unstable angina (0.05, 0.1, 0.2 or 0.3 mg/kg/hr, rH infusion) (Topol *et al.*, 1994).

Based on the results from the phase I and II trials, a number of major, multicenter phase III trials were organized, such as the Global Use of Strategies to Open Occluded Arteries (Gusto), Organization to Assess Strategies for Ischemic Syndromes (OASIS), Hirudin for Improvement of Thrombolysis (HIT), Thrombolysis In Myocardial Infarction (TIMI) and Hirudin in a European Restenosis Prevention Trial Versus Heparin Treatment in PTCA Patients (HELVETICA). The results from some of these studies have not been as encouraging as those of the phase I and II trials. The Gusto IIa trial had to be stopped due to the excessive hemorrhagic stroke incidences in patients with ischemic coronary syndromes tested with rH, when compared with heparin. rH and heparin were used at i.v. doses of 0.6 mg/kg plus 0.2 mg/kg/hr infusion, and 5000 U plus 1000- to 3000 U/hr infusion, respectively (Aylward, 1993; Gusto IIa investigators, 1994). Excessive intracranial hemorrhage was also found in the rH treated group in the HIT-III study, where patients with acute myocardial infarction were treated with rH. rH was used at an i.v. bolus

dose of 0.4 mg/kg and 0.15 mg/kg/hr infusion (Neuhaus *et al.*, 1994).

In the TIMI-9A trial, intra- and non-intracranial bleeding were observed in rH treated groups, when used as an adjunct to thrombolysis in myocardial infarction, at an i.v. bolus dose of 0.6 mg/kg and 0.2 mg/kg/hr infusion (Antman *et al.*, 1994). A new TIMI trial (TIMI-9B) has been configured with a lower dose of 0.1 mg/kg (bolus) and 0.1 mg/kg/hr (infusion).

The results from the major phase III trials have led to the design of newer clinical trials using reduced doses of rH. The subjects in these trials were patients with the target disease, involving serious cardiovascular defects. It may be speculated that the clearance of rH in these patients may be reduced due to possible renal malfunctions accompanying such major situations. Most of the above clinical trials employ the use of the activated partial thromboplastin time (APTT) to monitor rH activity. It may be useful to measure the absolute levels of rH in plasma and urine of such patients. Several newer studies are in progress to determine the efficacy of this agent in the prevention of post-surgical DVT and different forms of arterial thrombosis.

Hirudin Related Agents

Structure-activity relationship studies with hirudin have led to the development of related agents that are direct inhibitors of thrombin or have antiplatelet activity. A derivative of hirudin, known as **hirulog**, is composed of the terminal 12 amino acid residues of hirudin, which bind to the thrombin

anion-binding exosite, and the sequence: D-Phe-Pro-Arg-Pro, capable of binding to the thrombin catalytic site. A linker segment of glycyl residues is used to bridge the above two peptide fragments (Witting *et al.*, 1992; White, 1993; Johnson, 1994). Hirulog, similar to hirudin, is a bivalent thrombin inhibitor, that binds to thrombin with high affinity (K_i values in the nanomolar range). Hirulog is being investigated for clinical use in unstable angina pectoris (Sharma *et al.*, 1993) and prevention of restenosis after coronary angioplasty (Topol *et al.*, 1993).

Hirugen is another synthetic derivative of hirudin that is composed of the terminal dodecapeptide region of hirudin. Hence, hirugen binds to the anion-binding exosite region of thrombin. However, the *in vivo* antithrombotic activity of hirugen was found to be considerably weaker than hirudin and hirulog (Kelly *et al.*, 1992), and therefore, it is not being investigated in clinical studies (Lefkovits and Topol, 1994).

Hirudisins are derivatives of hirudin, that are being investigated for their antiplatelet activity (Knapp *et al.*, 1992). Hirudisins were developed by incorporation of an Arg-Gly-Asp-Ser (RGDS) sequence into the finger-like domain of hirudin (residues 27 to 40). These peptides have the ability to inhibit thrombin-independent platelet aggregation and activation observed in arterial thrombosis via the glycoprotein II_b/III_a receptor (Johnson, 1994).

A chemical conjugate of rH with two molecules of polyethylene glycol (PEG) has been shown to possess the main characteristic of hirudin, i.e.,

selective thrombin inhibition. In addition, **PEG-hirudin** has been shown to have extended duration of antithrombotic action (up to 24 hours), when compared with rH (Rübsamen *et al.*, 1994). Another chemical conjugate of dextran with hirudin, known as **dextran-hirudin**, has been shown to exhibit a lower volume of distribution (25 to 36 mL) and a longer half-life ($\approx$ 6 hours) in rats, when compared with free hirudin (Markwardt *et al.*, 1990).

Other Site-Directed Antithrombotic Agents

There is considerable interest in the development of antithrombotic drugs that have direct inhibitory action on thrombin. Thrombin plays a crucial role in platelet activation and thrombus formation that lead to coronary artery syndromes (Lefkovits and Topol, 1994). Besides hirudin, hirulog, hirugen and PEG-hirudin, there are other site-directed thrombin inhibitors that are currently undergoing investigation, such as **efegatran**, **argatroban**, and **PPACK**. Efegatran is a tripeptide (D-methyl-phenylalanyl-prolyl-arginal) that was found to exhibit dose dependent anticoagulant effects in animal studies, and is currently undergoing phase I human studies (Jackson, 1994).

(2R,4R)-4-methyl-1-{N^2-[(RS)-3-methyl-1,2,3,4-tetrahydro-8-quinolinesulfonyl]-L-arginyl}-2-piperidinecarboxylic acid hydrate, or argatroban, is a synthetic direct thrombin inhibitor that binds at the apolar-binding site of thrombin (Lefkovits and Topol, 1994: Plachetka, 1994). Argatroban was found to exhibit significant correlations between dose and

anticoagulant effects in animal models (Plachetka, 1994). PPACK (D-phenylalanyl-L-prolyl-L-arginyl chloromethyl ketone) is yet another direct thrombin inhibitor (irreversible) that binds to the catalytic site of thrombin (Powers and Kam, 1992). PPACK's *in vivo* activity has been shown to be attenuated due to reactions with other plasma constituents (Hauptmann and Markwardt, 1992).

CHAPTER II

STATEMENT OF PURPOSE

The primary purpose of this dissertation was to investigate the pharmacokinetics and pharmacodynamics of recombinant hirudin variant 2 (rHV2) in valid animal models. To accomplish this, anticoagulant, antithrombin (anti-IIa) and enzyme-linked immunosorbent assay (ELISA) methods were developed and optimized.

Hirudin is reportedly the most potent and specific natural inhibitor of thrombin (factor IIa) that was used in ancient medicine as an anticoagulant in the form of dry preparations and aqueous extracts from the medicinal leech, *Hirudo medicinalis*. More recently, the development and production of recombinant forms of hirudin variants using biotechnology have brought hirudin back into the focus of research interest again. A comprehensive review of published literature on rH with regard to structure, mechanism of action, available data on pharmacokinetics, assay methods and potential clinical uses, is included as part of this dissertation.

rHV2 investigated in this dissertation, was prepared by recombinant technology utilizing a yeast expression vector at Transgene, Strasbourg, France, and was provided by Dr. J.-P. Maffrand (Sanofi Recherche, Toulouse,

(France). This agent was studied in three animal species: rabbits, rats and dogs using two different routes of administration: i.v. (bolus) and s.c. administration. Until now, the pharmacokinetic/pharmacodynamic relationship of natural hirudin and rH has not been fully investigated, despite the efforts of a few groups of researchers to characterize the absorption, distribution, metabolism and elimination of this drug in human and animal subjects. Most pharmacokinetic studies have used an indirect method to analyze plasma concentrations of natural hirudin and rH, for example, thrombin clotting time (Markwardt *et al.,* 1989) or chromogenic anti-IIa assay (Markwardt *et al.*, 1982, 1984, 1988a, 1988b; Nowak *et al.,* 1988), with the exception of Bichler *et al.* (1988), who have used a radioimmunobioassay (RIBA) to directly measure concentrations of natural hirudin in plasma. A specific ELISA method has been developed for the studies reported in this dissertation for the measurement of absolute concentrations of rHV2 in plasma samples from animal models.

In addition, the *in vitro* and *ex vivo* pharmacodynamics of rHV2 have been studied using calcium-thrombin time ($Ca^{+2}TT$), activated partial thromboplastin time (APTT) and a chromogenic anti-thrombin assay. *In vivo* pharmacodynamics of rHV2 has been profiled using a modified rabbit jugular vein stasis thrombosis model (Wessler *et al.,* 1959; Fareed *et al.,* 1985). The hemorrhagic effects of rHV2 have been studied using a modified rabbit ear bleeding model (Cade *et al.,* 1984; Fareed *et al.,* 1985). These studies

provide crucial information on the relationship between antithrombotic and hemorrhagic actions of this agent. Pharmacodynamic parameters, bioavailability profile and other pharmacokinetic parameters have been compared between the two routes of administration using appropriate statistical methods.

New data on the status of renal function in rats after i.v. and s.c. administration of rHV2 have been provided in this dissertation. A multiple dosage study in dogs has been included to obtain more information on the *ex vivo* antithrombin properties of rHV2. Moreover, the hematological and blood chemistry profiles after repeated administration of rHV2 in dogs for a period of one week, are described.

A systematic and integrated approach to study the pharmacokinetics and pharmacodynamics of rHV2 has not been made to date. A direct approach to study pharmacokinetics by using absolute concentration measurements from the ELISA method has helped to differentiate between true pharmacokinetics from previously used indirect pharmacodynamic methods. The results from these experiments validate the difference between functional and immunological methods to monitor rHV2 after various dosage regimens in the three animal species.

The experimental methods used in this investigation represent optimized and pharmacologically valid experimental models to obtain much needed objective data on the pharmacokinetic and pharmacodynamic profile of this

recombinant antithrombotic agent. The data generated in this dissertation will be valuable in the design of newer clinical trials for i.v. and s.c. indications of this new anticoagulant drug. These studies will also serve as a model for obtaining valid pharmacological data on other recombinant antithrombotic drugs.

CHAPTER III

MATERIALS, METHODS AND EXPERIMENTAL PROTOCOLS

Materials

Recombinant Hirudin (rH)

Recombinant hirudin (rH) used in all *in vitro* and *in vivo* experiments in this dissertation was kindly supplied by Sanofi Recherche, Toulouse, France. According to the supplier, this variant of rH was recombinant hirudin variant 2 (rHV2) with a lysine residue in position 47 (rHV2-Lys47). This information was later reconfirmed by results from Drs. A. Patthy and S. Bajusz (Institute for Drug Research, Budapest, Hungary) (Appendix II). Henceforth, rH used in all experiments will be designated as "rHV2". A single batch of rHV2 was used in all studies i.e. ref no. SR 29010 and batch no. RHE 15, manufactured at Transgene, Strasbourg, France. As per the manufacturer's declaration (Appendix II), batch no. RHE 15 had been produced using an yeast expression vector. The specific activity and molecular weight of this batch have been specified by the manufacturer to be 14,495 ATU/mg and 6,906 daltons, respectively. Impurities, as determined by exclusion chromatography have been specified to be less than 0.8%.

Thrombin

Human thrombin (no. *a*362) was kindly provided by Dr. John Fenton II (New York State Department of Health, Albany, NY). Henceforth, this thrombin will be referred to as "Fenton *a*362" or "*a*362" thrombin, which has a specific activity of 3007.22 NIH units/mg. *a*362 thrombin is composed primarily of *a*-thrombin (95.72%), with trace amounts of ß-thrombin (2.37%) and *y*-thrombin (1.91%). *a*362 is free of contamination from HIV and hepatitis virus.

Reagents and Kits

0.025 M calcium chloride and 0.02 M calcium chloride solutions were obtained from Organon Teknika (Parsippany, NJ) and Baxter Health Corp., Dade Division (Miami, FL), respectively. Activated partial thromboplastin time (APTT) reagents were procured from Organon Teknika (Parsippany, NJ) and Baxter Health Corp., Dade Division (Miami, FL). Chromogenic substrate (Spectrozyme® TH) required for the amidolytic assay for thrombin was obtained from American Diagnostica (Greenwich, CT). Reagents required for Tris-EDTA buffer i.e. tris(hydroxymethyl)aminomethane (Trizma®), PEG 6000 (polyethylene glycol 6000), Na_2EDTA (Disodium ethylenediaminetetraacetic acid) and sodium chloride were all obtained from Sigma Chemical Co. (St. Louis, MO). Bovine aprotonin (6120 KIU/mg) was obtained from Pentapharm AG, Basel, Switzerland. 0.9% Sodium Chloride Irrigation Solution, USP, and

Sterile Water for Irrigation, USP, obtained from Baxter Healthcare Corp., Deerfield, IL, were used as normal saline and distilled water (free of metal contaminants), respectively.

For thrombogenic challenge in the modified stasis thrombosis model in rabbits, Factor Eight Inhibitor Bypass Activator (FEIBA®) was obtained from Osterreichisches Institut fur Haemoderivate GES.M.B.H. (subsidiary of IMMUNO AG, Vienna, Austria). A competitive ELISA based kit (Asserachrom® Hirudin) for the quantitation of hirudin antigen was obtained from Serbio Research Laboratories, Gennevilliers, France. For clearance studies, ^{3}H-Inulin was obtained from NEN/Dupont (Boston, MA) and liquid scintillation cocktail (Ecolite®) was obtained from ICN Biomedical, Inc. (Costa Mesa, CA).

Xylazine (Rompun®, Miles, Inc., Shawnee Mission, KS) and ketamine hydrochloride (Ketaset®, Aveco Co., Inc., Fort Dodge, IA) were used to anesthetize rabbits. Sodium pentobarbital and phenytoin sodium solution (Beuthanasia®-D, Schering-Plough Animal Health Corp., Kenilworth, NJ) were used for rabbit euthanasia. Halothane (Burns Veterinary Supply, Glenview, IL) was used via a dispenser (Fluotec® Mark 2, Cyprane Ltd., Keighley, England) to induce light anesthesia in rats. Rats were restrained using Decapicones®, Braintree Scientific, Inc., Braintree, MA.

Major Instruments

Major instruments used in the experimental protocols reported in this

dissertation include Fibrometer®s (Becton Dickinson and Co., Rutherford, NJ) for determination of clotting times, Beckman DU®-7 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) and ACL®300 Plus (Instrumentation Laboratory, Lexington, MA) for chromogenic substrate assays, Ultra Wash® microplate washer (Dynatech Laboratories, Inc., Chantilly, VA) and Dynatech MR 700® spectrophotometric reader (Dynatech Laboratories, Inc., Chantilly, VA) for the ELISA method, and a liquid scintillation counter (Minaxi Tri-Carb® 4000 series, Packard Instrumental Company, Downer's Grove, IL) for clearance studies. Some of the later experimental protocols utilized a combined washer and reader in the ELISA method (Dynatech MR 7000®, Dynatech Laboratories, Inc., Chantilly, VA). Serum chemistry profile, urinalysis and hematological profile were performed at the Clinical Laboratory Division, Department of Pathology, Loyola University Chicago (Paramax® 720ZX, Automated Chemistry Analyzer, Baxter Health Corp., Dade Div., Miami, FL; CX®3, Synchron Clinical System, Beckman Instruments, Inc., Brea, CA and Coulter® Stacker, Coulter Diagnostics, Hialeah, FL, respectively).

IBM compatible personal computers linked to printers (LaserJet III or IV, Hewlett Packard, San Diego, CA) were available for word processing, pharmacokinetic analysis, statistical analysis and graphics.

Animals

All animals were allowed an acclimation period of at least four days after

arrival in the Animal Research Facility, Loyola University Chicago, before initiation of experiments. Male New Zealand White rabbits (2.5-3.0 kg; LSR Industries, Inc., Union Grove, WI) were used in the modified rabbit jugular vein stasis thrombosis model, modified rabbit ear bleeding model and pharmacokinetic/ pharmacodynamic studies. Adult, male mongrel dogs (25-30 kg; Teays River Valley, Upland, IN) were used in pharmacokinetic and pharmacodynamic studies. Male Sprague-Dawley rats (250-300 g; Harlan, Indianapolis, IN) were used in pharmacokinetic and clearance studies. All animals used in the studies were used in accordance with the guidelines set forth by the Institutional Animal Care and Use Committee (IACUC)., Loyola University Chicago.

Pooled Plasma, Serum and Whole Blood Preparations

Blood bank plasma (BBP). BBP was obtained from the Blood Bank, Department of Pathology, Loyola University Chicago. This plasma was made from blood donated by human volunteers who were tested to be free of acquired immune-deficiency syndrome (AIDS) and hepatitis B virus. The fresh, frozen plasma packs, not more than 30 days old, were thawed at 37°C, pooled, aliquoted in volumes of 10 mL each and frozen at -70°C for a period of not more than two months. Pooled BBP was thawed at 37°C prior to use.

Normal rabbit plasma (NRP), normal rat plasma (NRatP) and normal dog plasma (NDP). Guidelines of IACUC, Loyola University Chicago were followed

in the preparation of pooled plasma from dogs, rabbits and rats. NRP, NRatP and NDP were made from normal male rabbits, rats and dogs, respectively.

Blood was collected from 10-12 conscious dogs via standard venipuncture of the forelimb vein using a Butterfly® (21 X 3/4, 12" tubing) infusion set (Abbott Hospitals, Inc., North Chicago, IL). Polypropylene syringes were used, with the initial 2-3 mL of blood being discarded. 4.5 mL of drawn blood were added to siliconized glass tubes (Vacutainer®, Becton Dickinson VACUTAINER Systems, Rutherford, NJ) containing 0.5 mL of 0.129 M (3.8%) sodium citrate solution. The collected blood was gently mixed with the anticoagulant and then centrifuged (Beckman GPR Centrifuge, Beckman Instruments, Inc., Fullerton, CA) at 2,500 rpm and 4°C for 20 minutes. The supernatant plasma was separated using polypropylene transfer pipettes and pooled to obtain a pooled, platelet-poor NDP. The NDP was stored in 1 mL aliquots at -70°C until use.

Cardiac puncture under xylazine- and ketamine-induced anesthesia was used to draw blood from 10-12 rabbits. The plasma was separated, pooled to obtain NRP and stored as described above.

Cardiac puncture under light halothane-induced anesthesia was used to draw blood from 8-10 rats. The blood was processed for plasma separation (NRatP) as described above.

Rat serum. For the measurement of creatinine and inulin clearances, blood (0.5 mL) was drawn from male Sprague-Dawley rats via tail bleeding using Bard-Parker® scalpel (no. 11, Becton-Dickinson AcuteCare, Franklin Lakes, NJ) into empty 1.5 mL microtubes (Sarstedt, Newton, NC). The blood was incubated at room temperature to activate clotting for at least an hour until serum separated. The clotted blood was centrifuged (Eppendorf® Microcentrifuge, Model 5415C, Brinkman Instruments, Inc., Westbury, NY) at 2,500 rpm for 20 minutes and the supernatant serum was separated and stored at -70°C until ready for use.

Dog and rat whole blood. Blood drawn from dogs (2 mL) or rats (0.5 mL) as described above was collected into Vacutainer® blood collection tubes or Microtainer® (with EDTA) obtained from Becton Dickinson VACUTAINER Systems, Rutherford, NJ. The tubes were tilted gently for mixing and stored at 4°C for not more than 6 hours until use to obtain hematological profiles.

Methods

Global Clot-based Assays

Activated Partial Thromboplastin Time (APTT)

APTT is a screening test for the intrinsic pathway of coagulation, and is used in monitoring heparin therapy. It is based on the measurement of clotting time after plasma has been activated with a platelet substitute

(phosholipid), silica and calcium chloride. Two different types of APTT reagents were used - APTT (Dade) and APTT (OT) obtained from Baxter Healthcare Corp., Dade Division, and Organon Teknika, respectively. 100 μL of APTT reagent were added to 100 μL of plasma sample (prewarmed at 37^{o}C), and incubated at 37^{o}C for 5 minutes (OT reagent) or 3 minutes (Dade reagent). The time taken for the sample to clot was determined using a Fibrometer® after addition of 100 μL of 0.025 M (GD/OT) or 0.02 M (Dade) $CaCl_2$ solution, respectively.

Thrombin Time (TT)

TT measures the time taken by thrombin to convert fibrinogen to fibrin. 200 μL of test plasma were warmed at 37^{o}C for 3 minutes and 100 μL of thrombin (Fenton α362, reconstituted in normal saline to give 5, 10 or 20 NIH units depending on the test) were added to initiate clotting. As before, the clotting time was measured using a Fibrometer®.

Calcium Thrombin Time (Ca^{+2}TT)

Both native hirudin as well as rH, being highly potent thrombin inhibitors, exhibit strong anti-IIa activity at very low concentrations. As a result, the clotting time, as monitored by TT, reaches beyond 300 seconds, which is the upper limit of sensitivity of the Fibrometer®. Hence, modified thrombin time tests were developed, where clotting was initiated by using a mixture of thrombin and 0.025 M $CaCl_2$ solution. This test was optimized in the

Hemostasis Research Laboratories, Loyola University Chicago, and was designated as calcium-thrombin time (5, 10, 20, or 40 NIH units, depending on the potency of thrombin). In this assay, thrombin (α362) was reconstituted with 0.025 M $CaCl_2$ solution to give 5, 10, 20 or 40 calcium-thrombin units. 100 μL of calcium-thrombin reagent were added to 200 μL of prewarmed (37°C, 3 minutes) test sample and the clotting time was determined using a Fibrometer®.

Δ 215-225 Method for Protein Determination

The Δ 215-225 method (Chaykin, 1966) was used to determine total protein content in batch no. RHE 15 of rHV2 supplied by Sanofi Recherche. rHV2 solutions were made in Sterile Water for Irrigation, to obtain concentrations of 25, 50 and 100 μg/mL. The optical densities of this series of rHV2 solutions were read at 215 and 225 nm, using a spectrophotometer (Beckman DU®7). The protein concentration in each sample was calculated by the formula (Chaykin, 1966):

μg/mL protein = (Δ 215-225) x 144.

The factor (144) has been previously determined and found to be identical on Beckman DU spectrophotometers (Waddell, 1956). Appropriate bovine serum albumin control was used.

Chromogenic Anti-IIa Assay

In this biochemical assay, the decrease in enzyme (thrombin or factor IIa)

activity produced by the inhibitor (rH) was determined by measuring the decrease in absorbance of the free chromophore (p-nitro aniline, pNA) per unit time at 405 nm. At excess substrate concentrations, the rate of decrease in absorbance was linearly related to the concentration of rH. The substrate used was Spectrozyme®TH (H-D-hexahydrotyrosyl-L-alanyl-L-arginine-p-nitroanilide diacetate salt). An Automated Coagulation Laboratory system (ACL® 300 Plus) or a spectrophotometer (DU®7, Beckman Instruments) were used to perform the chromogenic anti-IIa assay.

The ACL® 300 Plus system has a rotor capable of analyzing 16 samples at one time. 100 μL of α362 thrombin (5, 10 or 20 U/mL, depending on the test system, in tris-EDTA buffer, pH = 8.4) were added to 10 μL of sample, incubated at 37°C for 1 minute followed by the addition of 40 μL of Spectrozyme®TH (1 μM/mL solution in Sterile Water for Irrigation, USP), in an automated fashion. Absorbances were read at 405 nm. The system was linked to an IBM compatible personal computer that was equipped with software to calculate the rate of change of absorbance with time. Control samples were analyzed using appropriate plasma with no rHV2.

While using the DU®7 spectrophotometer, 400 μL of tris-buffer (pH = 8.4) were incubated with 25 μL of test sample in a cuvette at 37°C for 1 minute, followed by addition of 25 μL of 5, 10 or 20 NIH units/mL of α362 thrombin. After mixing and incubating at 37°C for an additional minute, 50 μL of Spectrozyme®TH (1 μM/mL) were added. The rate of change of absorbance

at 405 nm was noted.

The composition of tris-EDTA buffer used in both methods was as specified below:

Sodium Chloride (175 mM)	-	10.227 g
Tris (50 mM)	-	6.055 g
Na_2EDTA (7.5 mM)	-	2.792 g
Aprotonin (1 μg/mL)	-	1.0 mg
PEG 6000 (0.25%)	-	2.5 g
Distilled Water	-	1000.00 mL (q.s.)

The above ingredients were dissolved in distilled water and pH adjusted to 8.4. Tris buffer was stored at 4°C until use (within 7 days).

Enzyme-Linked Immunosorbent Assay (ELISA)

Asserachrom® Hirudin ELISA kits (Serbio Research Laboratories, Gennevilliers, France) were used to measure rHV2 concentrations in this dissertation. "Sandwich" and "competitive" ELISA methods for the measurement of rHV2 and other hirudin variants in biological fluids were developed and validated (Iyer *et al.*, 1995) in collaboration with Serbio Research Laboratories, Gennevilliers, France. The assay employed in this dissertation was of the competitive type. In this method, microwells from a 96-well microplate were precoated with hirudin antigen (native hirudin). Fifty μL of standard or test sample containing competing hirudin were added to

each well followed by addition of 200 μL of anti-hirudin antibodies, specifically raised in rabbits against rHV2 and labelled with horseradish peroxidase (HRP) enzyme. The microplate was incubated with slow agitation at room temperature for 1 hour during which a competition ensued between the test hirudin and coated hirudin for the antibody. After five successful washings, color development was achieved by addition of 200 μL of ortho-phenylene diamine (OPD) substrate in the presence of 10 μL of hydrogen peroxide (H_2O_2). Reaction was stopped after 6 minutes with addition of 50 μL of 3M sulfuric acid. Absorbances were read at 405 nm after a stabilization period of 10 minutes and were inversely proportional to concentration of rHV2 in test samples.

The linear range for this assay was 25 to 1000 ng/mL with an intra- and inter-assay variation of 3.9% and 6.8%, respectively (Iyer *et al.*, 1995). This required the dilution of test samples from pharmacokinetic experiments in animal models in assay dilution buffer (pH = 7.5) to bring final concentrations of rHV2 within the linear range. The standard curve was prepared from the same batch of rHV2, previously supplemented *in vitro* in the plasma system (NDP, NRP or NRatP) used, frozen at -70^{o}C and thawed just prior to use. It is to be noted that the standard curves were subjected to the same dilution steps as the test samples in order to eliminate any matrix effect from surrounding plasma system (Iyer *et al.*, 1995). Unknown concentrations of rHV2 in test samples were interpolated from a semilogarithmic plot of absorbances at

405 nm versus concentrations of standard. Figures 7 and 8 illustrate a schematic of the competitive ELISA method. It is most likely that this polyclonal antibody based ELISA method is capable of detecting metabolites of hirudin, which may or may not be active, and may be termed as "hirudin equivalents".

Rabbit Jugular Vein Stasis Thrombosis Model

A modified rabbit jugular vein stasis thrombosis model (Wessler *et al.*, 1959; Fareed *et al.*, 1985) was used to study the *in vivo* antithrombotic properties of rHV2. In this model, male New Zealand White rabbits (2.5-3.0 kg) were weighed and anesthetized with 20 mg/kg (i.m.) of xylazine (Rompun®) and 80 mg/kg (i.m.) of ketamine hydrochloride (Ketaset®). The rabbits were immobilized and prepared for surgery (neck area was shaved). Incisions were made from top of neck to bottom of sternum, and from the top and base of first incision toward each side to make flaps which would open and expose the underlying right and the left jugular veins. A fine-tip high temperature cautery (Accu-Temp®, Xomed-Treace, Jacksonville, FL) was used to cut the underlying, attached tissue and prevent any bleeding. Y-shaped segments of the two jugular veins were thus isolated, and the test dose of rHV2 was injected i.v. (via marginal ear vein) or s.c. (in the abdominal area) followed by a circulation time of 5 minutes or 2 hours, respectively. Figure 9 illustrates the two isolated Y-shaped segments of jugular veins. Table 3

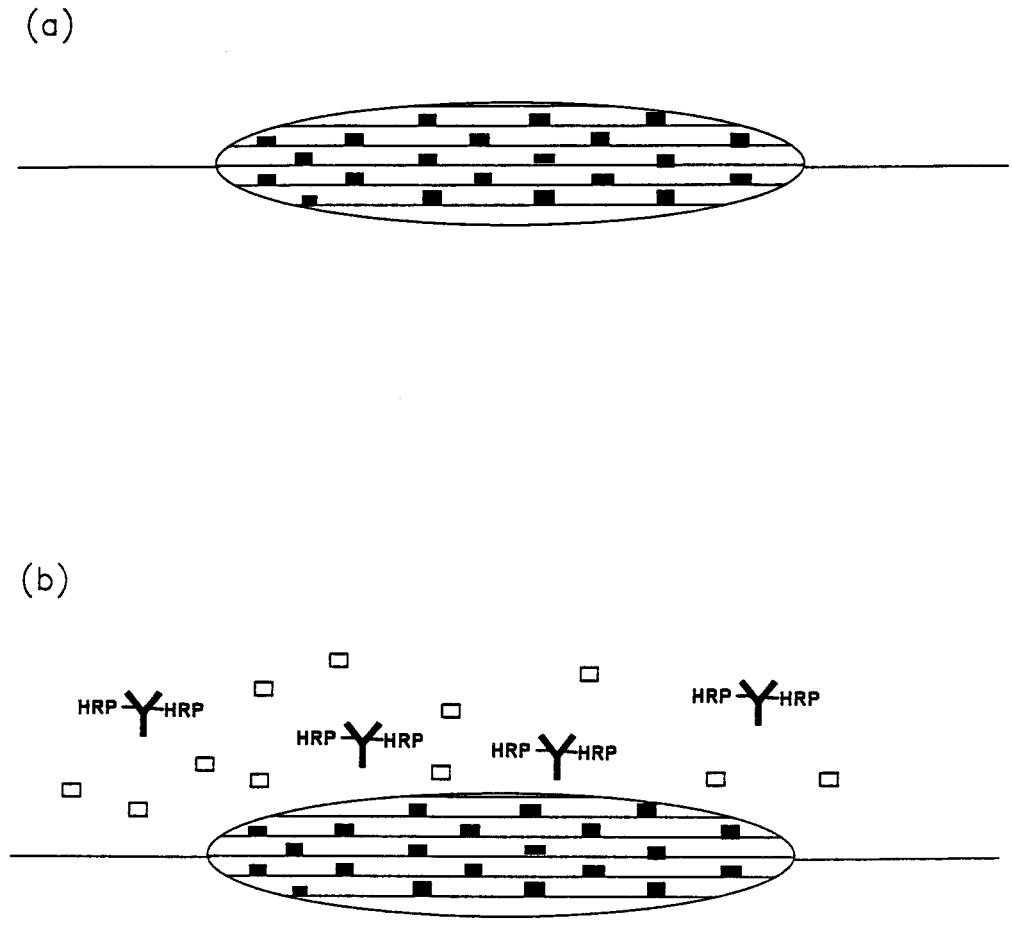


Figure 7. Scheme of competitive ELISA method (part I). Microplate (a) is pretreated with hirudin antigen (■) followed by addition (b) of competing hirudin (□) in presence of horseradish peroxidase (HRP) labelled antibody (**Y**).

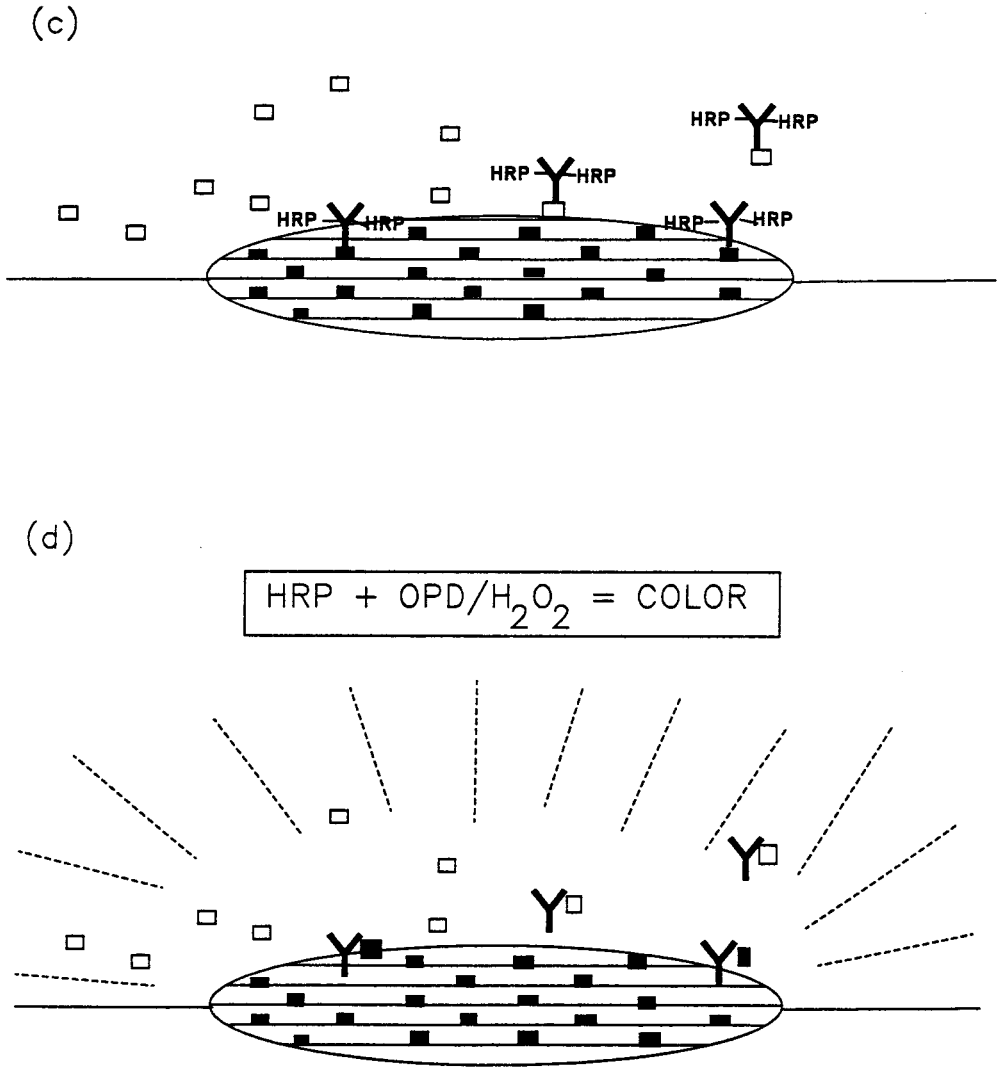


Figure 8. Scheme of competitive ELISA method (part II). A competition ensues between precoated hirudin antigen (■) and competing hirudin (□) during incubation (c) at room temperature. Absorbance is read at 492 nm after color development (d) due to addition of OPD/H_2O_2.

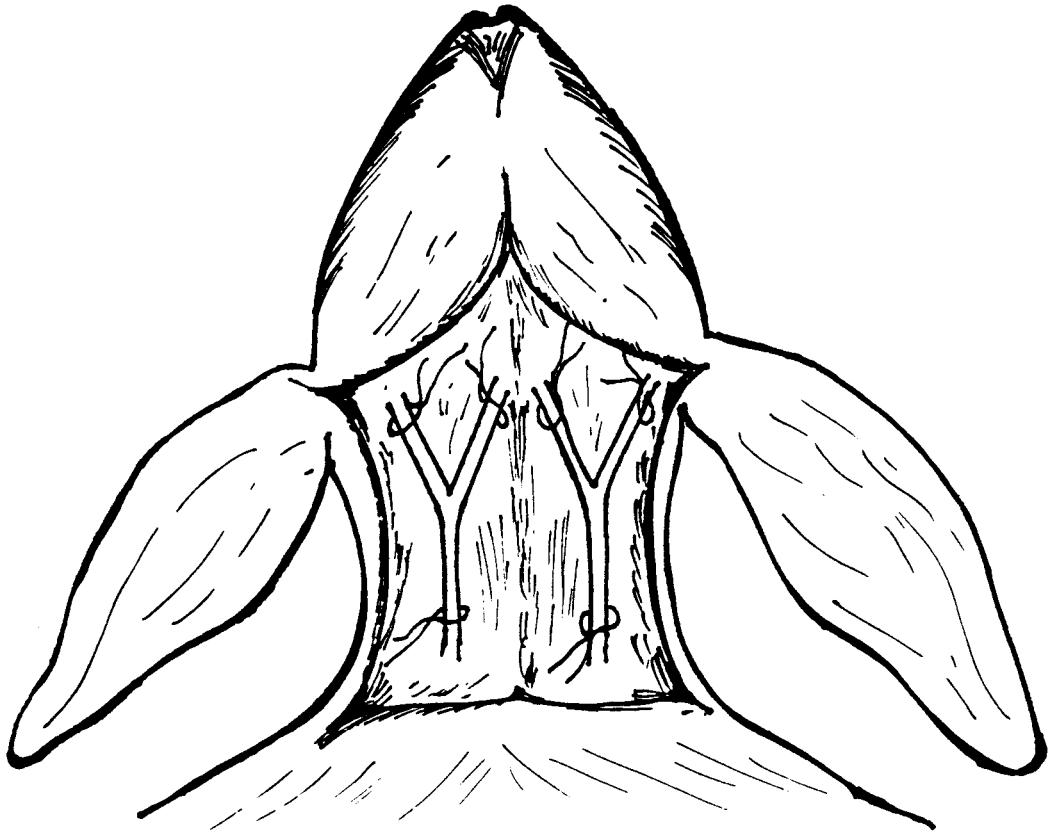


Figure 9. An illustration of the modified Wessler's rabbit jugular vein stasis thrombosis model. Y-shaped jugular vein segments are isolated and prepared for ligation.
(Modified, by permission, from Walenga, "Factor X_a inhibition in mediating antithrombotic actions: application of a synthetic heparin pentasaccharide". Ph.D. diss., University of Paris, 1987, p82.)

lists the time schedule used in the modified rabbit jugular vein stasis thrombosis model.

At the end of the specified circulation time of rHV2 or saline, FEIBA® (factor VIII inhibitor bypass activator-25 U/kg) was administered as a thrombogenic challenge (via marginal vein) and allowed to circulate for 20 seconds. The jugular vein segments were ligated using surgical silk and thereby a stasis of blood flow was produced.

After 10 minutes, any clots formed in one segment (left side) were removed, placed in a dish (S/P® Brand diSPo® Petri Dish, Baxter Healthcare Corp., McGaw Park, IL) with normal saline, visually analyzed and graded to give a clot score (see below, Walenga, 1987). After another 10 minutes, any clots formed in the other segment (right side) were similarly evaluated. Blood samples were drawn via standard carotid artery catheterization at baseline, prior to the thrombogenic challenge ("post-drug"), and 6 minutes after the thrombogenic challenge ("post-FEIBA®"). The samples were analyzed using different clot-based tests and chromogenic anti-IIa assay. Control experiments were performed using 0.9% saline instead of rHV2. The rabbits were sacrificed by i.v. injection of Beuthanasia®-D solution. In the time-course experiments, the drug was allowed to circulate for varying times after i.v. and s.c. administration before stasis was performed.

Clot-Score Grading System (Walenga, 1987)

The clot-score grading system is illustrated in Figure 10. The clots obtained were graded on a scale from 0 to +4, where "0" score indicated no clot formation with blood in a fluid state, "+1" represented mostly fluid blood with a few minute clots, "+2" was a larger amount of slightly bigger clots, "+3" indicated more definite clots (fully formed or broken to pieces forming a Y shape) with some fluid blood and "+4" clot represented a firm, large, solid Y-shaped clot with no surrounding blood. The grading of clots was always performed by an additional, independent observer.

Rabbit Ear Bleeding Model

The rabbit ear bleeding model (modified from Cade *et al.*, 1986) was used to study the hemorrhagic effects of rHV2. Male New Zealand White rabbits (2.5-3.0 kg) were anesthetized by i.m. administration of 10 mg/kg of Rompun® and 80 mg/kg of Ketaset®. The test rHV2 dose was administered intravenously via the marginal ear vein (right ear), or subcutaneously in the abdominal area. One ear (usually left, opposite to the ear with site of i.v. injection) was immersed in a saline bath (one liter) at 37°C for 5-10 seconds, and using transillumination, an area between the central ear vein and the marginal ear vein was selected that was free from major blood vessels. The drug was allowed to circulate for 5 minutes and 1 hour after i.v. and s.c. administration, respectively. Using a Bard-Parker® surgical blade

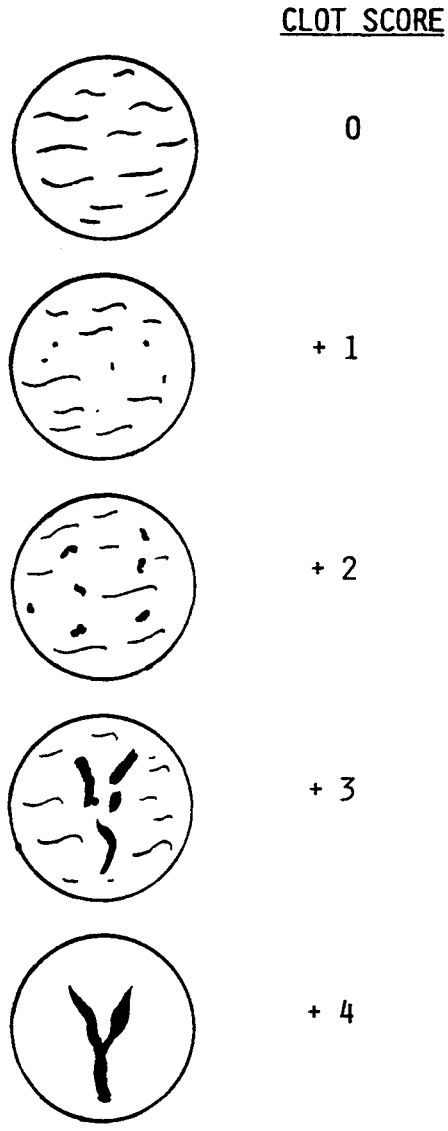


Figure 10. Clot-score grading system used in the modified rabbit jugular vein stasis thrombosis model. Examples of "O" to "+4" clots.
(Modified, by permission, from Walenga, "Factor X_a inhibition in mediating antithrombotic actions: application of a synthetic heparin pentasaccharide". Ph.D. diss., University of Paris, 1987, p83.)

TABLE 3

TIME SCHEDULE USED IN THE MODIFIED RABBIT JUGULAR VEIN STASIS THROMBOSIS MODEL

Time (I.V.)	Procedure	Time (S.C.)
	Anesthetize rabbit, shave neck area, expose Y-shaped jugular vein segments	
0 min ---->	Baseline blood draw Inject rHV2	<----0 min
5 min ----> (Circulation Time)	Post-drug blood draw. Inject FEIBA® Ligate vein segments after 20 seconds	<----120 min (Circulation Time)
11 min --->	Post-FEIBA® blood draw	<----126 min
15 min --->	Isolate one segment Grade clot	<----130 min
25 min --->	Isolate other segment Grade other clot	<----140 min
	Euthanize rabbit	

(Becton-Dickinson AcuteCare, Franklin Lakes, NJ), five uniform full thickness incisions were made in the selected area. An example of the incisions is illustrated in Figure 11. Immediately after making the incisions, the ear was immersed in the saline bath (stirred constantly) for 10 minutes. Five uniform incisions were similarly made in the other ear (right ear) at the end of 15 minutes and 3 hours after i.v. and s.c. administration, respectively. Blood loss was measured for another 10 minutes in each case, in a fresh saline bath. The blood from each ear was thus collected in saline in one liter bottles and the total number of red blood cells per liter were counted using a Bright Line® hemacytometer (0.1 mm deep, American Optical, Buffalo, NY). Control experiments were performed using saline instead of rHV2. Table 4 illustrates the time-schedule used in the modified rabbit ear blood loss model.

Figure 11. An illustration of the modified rabbit ear bleeding model. Five uniform incisions are made in an area free of blood vessels.
(Modified, by permission, from Racanelli, "Biochemical and pharmacological studies on the interaction of protamine with heparins", Ph.D. diss., Loyola University Chicago, 1990, p332.)

TABLE 4

TIME SCHEDULE USED IN THE MODIFIED RABBIT EAR BLOOD LOSS MODEL

Time (I.V.)	Procedure	Time (S.C.)
	Anesthetize rabbit, visually select area of both ears for potential sites of incisions	
0 min---->	Inject rHV2	<----0 min
5 min---->	Make five uniform incisions in one ear, immerse ear in saline bath	<----60 min
15 min---->	Remove ear and collect RBCs in saline for counting	<----70 min
15 min---->	Make five uniform incisions in other ear, immerse ear in saline bath	<----180 min
25 min---->	Remove other ear and collect RBCs in saline for counting	<----190 min
	Put rabbit back in cage after bleeding ceases	

Experimental Protocols

In Vitro Studies

1. Construction of Concentration-Response Curves after *In Vitro* Supplementation of rHV2 in Plasma Systems

Calibration curves were constructed after *in vitro* supplementation of rHV2 in BBP, NDP, NRatP and NRP. The following tests were performed after supplementation of rHV2 in three individual replicates with each sample analyzed duplicate:

1. APTT (OT and Dade)
2. TT
3. $Ca^{+2}TT$
4. Chromogenic anti-IIa assay

In case of NRatP, only the chromogenic anti-IIa assay was performed, due to the limitation of the amount of blood that can be obtained from rats. rHV2 was supplemented in concentrations ranging from 0 to 10 μg/mL in the respective plasma systems, frozen at -70^{o}C and thawed just prior to use. Appropriate dilutions were made in respective plasma, for each assay. The used for the standard curves were frozen for approximately the same period of time as the samples from the pharmacokinetic /pharmacodynamic studies.

2. Determination of Specific Activity of rHV2 using a Thrombin Titration Method

Principle

One antithrombin unit (ATU) of rH is the amount that inhibits 1 NIH unit of thrombin.

Materials

Assay working buffer (pH = 8.4).

Triethanolamine HCl (M.W. = 185.65)	-	0.1 M or 18.6 g
NaCl (M.W. = 40)	-	0.2 M or 11.7 g
Distilled Water	-	1000 mL (q.s.)

Triethanolamine and NaCl were dissolved in 800 mL of distilled water and pH was adjusted to 8.4 with 1N NaOH. The volume was made up to 1000 mL.

Thrombin dilution buffer (pH = 7.9).

Tris (M.W. = 121.1)	-	0.05 M or 6.06 g
NaCl (M.W. = 40)	-	0.1 M or 5.84 g
PEG 6000	-	0.1 % or 1 g
Distilled Water	-	1000 mL (q.s.)

All ingredients were dissolved in distilled water to make 1000 mL and pH was adjusted to 7.9.

Thrombin. 235 μL (23.5 U) of 100 U/mL thrombin (Fenton α362, 2435.85 U/mL) were diluted with 765 μL of thrombin dilution buffer to make

1000 μL of 23.5 U/mL solution of thrombin in dilution buffer. 100 μL of this solution were used to give a final thrombin concentration of 1 U/mL in 2.35 mL of total assay volume in each cuvette.

Substrate. Spectrozyme® TH (H-D-hexahydrotyrosyl-L-alanyl-L-arginine-p-nitroanilide-diacetate salt, 5 μM/vial, American Diagnostica, CT) was reconstituted with 3.33 mL of distilled water to give a 1.5 μM/mL solution. 250 μL were used to give a final substrate concentration of 0.160 μM/mL.

Recombinant hirudin. Serial dilutions were made in normal saline from a 1 mg/mL solution of rHV2 to yield final assay concentrations of 1.59 to 69.1 ng/mL.

Procedure

In a polystyrene cuvette (pathlength = 1cm), buffer and sample were preincubated at 37°C for two minutes and mixed. α-thrombin was added, mixed and incubated at 37°C for one minute followed by addition of substrate. The O.D. variation per minute (Δ O.D./min) at 37°C was measured at 405 nm during a time period of one minute using a spectrophotometer (DU-7®, Beckman). Given below are the individual volumes of each constituent:

Buffer - 1975 μL

Sample - 25 μL

Mix and incubate at 37°C for two minutes.

Thrombin (2.35 U/mL) - 100 μL

Mix and incubate at 37°C for one minute.

Spectrozyme® TH - 250 μL

Total Volume - 2350 μL

The antithrombin response using each concentration of rHV2 was measured as "% inhibition of thrombin" in duplicate when compared with saline controls. The "residual thrombin activity" in each sample was plotted against rHV2 concentrations and specific activity of rHV2 was determined using the rHV2 concentration corresponding to a residual thrombin activity of zero. The specific activity was thus determined three different times followed by calculation of mean specific activity.

3. Determination of Protein Content of rHV2 by Δ 215-225 Method

Appropriate dilutions of rHV2 were made in Sterile Water for Irrigation to give concentrations of 25, 50, and 100 μg/mL. The absorbances of each solution were read at 215 and 225 nm using a spectrophotometer. The protein content in each sample was determined as described in the "METHODS" section.

In Vivo Studies

1. Assessment of Antithrombotic Profile of rHV2 using a Modified Jugular Vein Stasis Thrombosis Model in Rabbits - A Dose-Ranging Study

Procedure. Male, New Zealand White rabbits (2.5-3.0 kg) were used in

a modified Wessler's jugular vein stasis thrombosis model to evaluate the *in vivo* antithrombotic effects of rHV2 as described under "METHODS" section. A circulation time of 5 and 120 minutes was allowed after i.v. and s.c. administration of rHV2, respectively.

Sample size. A sample size of 5 rabbits was used for each dose including saline treated control rabbits.

Dosage.

I.V. - 0 (saline), 6.25, 12.5 and 25 μg/kg weight of each rabbit

S.C. - 0 (saline), 125, 250 and 375 μg/kg weight of each rabbit.

FEIBA® was used in a dose of 7.5 U/kg, i.v.

Blood sampling times.

Baseline, post-drug (5 minutes/i.v. and 120 minutes/s.c.) and post-FEIBA® (11 minutes/i.v. and 126 minutes/s.c.).

Ex vivo analysis. Clot scores were graded in a scale from 0 to +4 as described under "METHODS". Plasma samples were analyzed for rHV2 concentrations using competitive ELISA method. Coagulation tests, including APTT, TT, and Ca^{+2}TT as well as chromogenic anti-IIa assay were performed.

2.. Assessment of Hemorrhagic Activity of rHV2 using a Modified Rabbit Ear Blood Loss Model

Procedure. A modified rabbit ear bleeding model was used to evaluate

the bleeding profile of rHV2 as described under "METHODS". Hemorrhagic responses were measured in experimentally determined therapeutic and supratherapeutic doses for a period of 10 minutes from each ear as described under "METHODS". No restriction on food and water was placed prior to anesthesia of rabbits.

Sample size. A sample size of 5 rabbits was used for each i.v. or s.c. dose.

Dosage.

I.V. - 0 (saline), 0.025, 0.25, 0.5, 0.75, 1.0 and 2.5 mg/kg.

S.C. - 0 (saline), 0.375, 1.0, 2.5 and 5 mg/kg.

Circulation times.

I.V. - 5 minutes (one ear) and 15 minutes (other ear).

S.C. - 60 minutes (one ear) and 180 minutes (other ear).

3. Evaluation of Pharmacokinetics and Time Course of Antithrombotic Activity after rHV2 Administration in Rabbits

Procedure. The purpose of this experiment was to relate the time course of *in vivo* antithrombotic activity of rHV2 with time course of plasma concentrations of rHV2. Male, New Zealand White rabbits (2.5-3.0 kg) were used in a modified stasis thrombosis model to obtain a profile of *in vivo* and *ex vivo* antithrombin activity. No restriction on food and water was placed

prior to anesthesia of rabbits.

Sample size. Five rabbits were used for each circulation time after i.v. and s.c. administration of rHV2.

Dosage. A dose of 25 μg/kg, i.v. and 375 μg/kg, s.c. were selected after reviewing the results from the dose-ranging study in rabbits.

Circulation times.

I.V. - 5, 15, 30, 45, 60, 90 and 120 minutes.

S.C. - 30, 60, 120, 150, 180, 240 and 360 minutes.

Blood sampling times. Additional blood samples (4.5 mL, two draws from each rabbit) were drawn to estimate rHV2 pharmacokinetics:

I.V. - 3, 5, 9, 12, 15, 20, 25, 30, 35, 40, 45, 50, 60, 75, 90, 100 and 120 minutes.

S.C. - 5, 10, 20, 30, 45, 60, 75, 90, 110, 120, 135, 150, 180, 200, 220, 240, 300, 320 and 360 minutes.

Three post-drug blood draws were performed in each rabbit for every circulation time in the time course studies (composite grouping).

Ex vivo analysis. Plasma samples from each time point were subjected to similar analysis methods as described before.

4. Determination of Pharmacokinetic Characteristics and *Ex Vivo* Antithrombin Activity of rHV2 in Rats

Procedure. Male, Sprague-Dawley rats (250-300 g) were weighed and injected with increasing i.v. or s.c. doses of rHV2 via tail vein or in the abdominal area, respectively. 450 μL of blood were drawn into microtubes with 50 μL 3.8% sodium citrate solution, at specific time intervals via tail clipping method (Kraus, 1980, Luke *et al.*, 1991). After each blood draw, the tail ends were cauterized using an Accu-Temp®, high temperature cautery. Halothane (1% with oxygen, Kaczmarczyk and Reinhardt, 1975), used with a dispenser (Fluthane®) and maintained using a nose cone was utilized for induction of anesthesia during each blood draw. Rats were returned to their cages during periods between blood draws. No restriction on food and water was placed during study period. Microtubes with blood were centrifuged at 2,500 rpm at 4°C for 20 minutes, plasma separated and stored at -70°C until further use. The rats were sacrificed by administration (intra-cardiac) of 0.2 mL of Beuthanasia® solution.

Sample size. Eight rats were used in each sub-treatment group (see below).

Dosage. rHV2 dissolved in normal saline was administered in the following doses:

I.V. - 0.1, 0.4 and 0.5 mg/kg.

S.C. - 0.1, 0.5 and 1.0 mg/kg.

Blood sampling times. Three different time schedules (n = 8 rats, each) were used for each i.v. and s.c. dose (total = 24 rats per i.v. or s.c. dose) to cover a broader range of time period. The following were the time schedules used for each dose:

I.V.:

Schedule 1 - baseline, 3, 12, 25, 40, 60, 120 and 360 minutes.

Schedule 2 - baseline, 5, 15, 30, 45, 75, 180 and 480 minutes.

Schedule 3 - baseline, 9, 20, 35, 50, 90, 240 and 1440 minutes.

S.C.:

Schedule 1 - baseline, 30, 90, 130, 160, 190, 220, 300 and 400 minutes.

Schedule 2 - baseline, 60, 105, 140, 170, 200, 240, 320 and 480 minutes.

Schedule 3 - baseline, 75, 120, 150, 180, 210, 270, 360 and 1440 minutes.

A total of 144 rats were thus randomly assigned into the above composite groups. Equal volume ($\approx$ 0.5mL) of normal saline was injected i.p. for volume replenishment.

Ex vivo analysis. Plasma samples were analyzed for rHV2 concentrations using competitive ELISA method and antithrombin activity using chromogenic anti-IIa assay.

5. Evaluation of Renal Function after rHV2 Administration in Rats

Procedure. Male Sprague-Dawley rats (250-300g) were housed in individual metabolic cages and allowed an acclimation period of at least two days. Complete urine voiding was ensured using a halothane nose cone prior to drug administration. The test dose of rHV2 was injected via the tail vein (i.v.) or in the abdominal area (s.c.). An equal number of normal saline-treated control rats were used for each route of administration. Blood samples were drawn via tail bleed at baseline and at 24 hours for obtaining serum chemistry and hematological data. No restriction on food and water was placed during entire study period.

24 hours after drug (or saline) administration, a single dose ^{3}H-inulin clearance was performed. The rats were transferred from metabolic cages to individual, disposable bins and anesthetized with halothane nose cone. A bolus dose of ^{3}H-inulin (10 μCi) was administered via tail vein. Blood samples (200 μL) were drawn at regular intervals, serum separated, and stored at -70^{o}C. Prior to analysis, the serum samples were thawed, and the ^{3}H disintegrations per minute (dpm) were read after addition of 5 mL of Ecolite® to 10 μL of each sample using a counter (Minaxi®4000 series) for a period of 2.5 minutes/sample. At the end of the study, the rats were sacrificed with an intra-cardiac administration of Beuthanasia® solution.

Sample size. Eight rats were used in each treatment group (i.v. or s.c. dose with saline controls).

Dosage.

I.V. rHV2 - 0.5 mg/kg.

S.C. rHV2 - 1.0 mg/kg.

The above doses were selected from the results from pharmacokinetic experiments in rats, described above.

Blood sampling times. Blood samples were collected at baseline and 24 hours (1 mL) for preparation of whole blood and serum. After administration of ^{3}H-inulin, 200 μL of blood were collected at the following times:

Baseline, 5, 10, 20, 30, 45, 60, 75 and 90 minutes.

Ex vivo analysis. Serum samples were analyzed for ^{3}H disintegrations per minute using a scintillation counter. In addition, serum chemistry and hematological profiles were obtained.

6. Investigation of Pharmacokinetics and Pharmacodynamics of rHV2 after Single Dose Administration in Dogs

Procedure. Conscious, adult, male mongrel dogs (25 to 30 kg) were weighed and randomly treated with increasing doses of rHV2 dissolved in normal saline. The test dose (see below) was administered via the superficial saphenous vein (i.v.) or in the lower abdominal area (s.c.). Blood samples (9

mL) were obtained via standard venipuncture in the forelimb using Butterfly® (21 x 3/4, 12"tubing) infusion set (Abbott Hospitals, Inc., North Chicago, IL) at various time intervals as specified below. Blood samples collected in Vacutainers® were centrifuged at 2500 rpm at 4°C for 20 minutes and plasma samples from individual blood draws were separated and stored at -70°C until further analysis. No restriction on food and water was placed during the entire study period.

Sample size. A sample size of 6 dogs was used for each treatment (dose). The dogs were randomly assigned with treatment sequences.

Dosage. rHV2 was used in i.v. and s.c. doses as given below:

I.V. - 0.25, 0.5 and 1.0 mg/kg.

S.C. - 0.25, 0.5 and 1.0 mg/kg.

Blood sampling times. The following blood sampling times were used:

I.V. - baseline, 3, 6, 12, 18, 30, 60, 120, 360 and 1440 minutes.

S.C. - baseline, 15, 30, 45, 60, 90, 120, 150, 180, 240, 360 and 1440 minutes.

Ex vivo analysis. Plasma concentrations of rHV2 were determined using the competitive ELISA method. *Ex vivo* antithrombin activity was screened using chromogenic anti-IIa assay as well coagulation tests such as APTT (Dade), APTT (OT), TT (20 U/mL), and Ca^{+2}TT (10 and 20 U/mL).

7. Investigation of Pharmacodynamics of rHV2 after Repeated Administration in Dogs

Procedure. Conscious, adult, male mongrel dogs (25-30 kg) were administered with multiple i.v. or s.c. doses of rHV2 for one week at a dosing interval of every 24 hours. Blood samples were drawn at various time intervals and processed as described above.

Sample size. A sample size of 6 dogs was used for each treatment (i.v. dose or s.c. dose).

Dosage. A 1 mg/kg (i.v. and s.c.) dose was selected from the single dose studies described above.

Blood sampling times. The following time schedule was used for blood draws after the first dose:

I.V. - baseline, 3, 6, 12, 18, 30, 60, 120, 360 and 1440 minutes.

S.C. - baseline, 15, 30, 45, 60, 90, 120, 150, 180, 240, 360 and 1440 minutes.

The following time schedule was used for blood draws after administration of the second to the last dose:

I.V. - baseline, 5, 30 and 60 minutes.

S.C. - baseline, 120, 180 and 240 minutes.

Additional blood samples (2 mL) were obtained after each dose at baseline and 30 minutes (i.v.) or 120 minutes (s.c.) to obtain serum chemistry

and hematological profiles.

Ex vivo analysis. The plasma samples were subjected to the same tests to determine plasma concentrations and antithrombin activity of rHV2 as described in the single dose studies in dogs. In addition, serum samples and whole blood samples at specific time intervals were analyzed for chemistry and hematological profiles.

Data Analysis

Plasma concentrations of rHV2 were plotted against corresponding times using a graphics software (SigmaPlot®, Version 5.01, Jandel Corporation, Sausalito, CA). Various pharmacokinetic parameters were calculated using standard non-compartmental methods (Gibaldi and Perrier, 1982; Gibaldi, 1984; Rowland and Tozer, 1989) as shown below:

The maximum concentration of rHV2 reached in plasma (C_{max}) and time to reach C_{max} (t_{max}) were determined by visual examination of the graphs.

Elimination rate constant (k_e) of rHV2 was determined from the slope of the terminal portion of the plasma concentration-time curve.

Terminal half-life ($t_{1/2}$) of rHV2 was determined using the equation,

$$t_{1/2} = 0.693/k_e.$$

Area under the concentration-time curve up to the last blood sampling time (AUC_{0-t}) was estimated using the trapezoid rule and extrapolated

to infinity ($AUC_{0-\infty}$) using the formula,

$$AUC_{0-\infty} = AUC_{0-t} + C^*/k_e$$

where C^* was the concentration of rHV2 at the last sampling time (t^*). Area under the (first) moment curve up to the last sampling time ($AUMC_{0-t}$) was calculated from a plot of the product of drug concentration and time versus time. $AUMC_{0-\infty}$ was calculated from the formula,

$$AUMC_{0-\infty} = AUMC_{0-t} + C^*.t^*/k_e + C^*/(k_e)^2.$$

After single dose administration, mean residence time (MRT) of rHV2 or the time required to eliminate 63.2% of dose, was calculated as follows:

$$MRT = AUMC_{0-\infty}/AUC_{0-\infty}.$$

Bioavailability (F) of rHV2 after s.c. administration was determined from the following equation:

$$F = AUC_{0-\infty,\ s.c.}/AUC_{0-\infty,\ i.v.} \times Dose_{i.v.}/Dose_{s.c.}.$$

Systemic (total) clearance (Cl_{tot}) of rHV2 was calculated using the formula, $Cl_{tot} = Dose/AUC_{0-\infty}$ after i.v., and F X $Dose/AUC_{0-\infty}$, after s.c. administration.

Apparent volume of distribution (V_d) of rHV2 was determined using the formula,

$$V_d = Cl_{tot}/k_e.$$

To determine the inulin clearance parameter, the disintegrations per minute per minute (dpm) of ^3H of each blood sample were plotted (semilogar-

ithmic) versus time and the elimination rate constant of ^{3}H-inulin was determined from slope of terminal portion after linear regression. AUC_{0-t} was determined by trapezoidal rule and extrapolated to infinity ($AUC_{0-\infty}$) by the sum of AUC_{0-t} and $AUC_{t-\infty}$ (as described previously). Inulin clearance (Cl_{in}) was calculated from dose/$AUC_{0-\infty}$. This method has been previously validated and found to closely estimate the true glomerular filtration rate in rats (Luke *et al.*, 1991).

In the pharmacodynamic studies, each response was expressed as mean ± standard deviation (SD) and/or standard error of the mean (SEM). Responses were also plotted against time. Relationship between pharmacokinetics and pharmacodynamics were determined by correlation of time course of each response with time course of concentration in plasma.

Statistical Analysis

Appropriate statistical analytical tests were used to determine significance in differences between the various treatment groups (Zar, 1984). One-way analysis of variance (ANOVA) was performed to test for differences between two or more treatment groups on different animals, followed by multiple comparison tests such as Tukey test. One-way and two-way repeated measures ANOVA tests were performed in the multiple dose experiments in dogs (Wilkinson, 1990). For non-parametric analysis, Kruskal-Wallis one-way ANOVA was performed, followed by multiple comparison

using Mann-Whitney test.

In the case of treatments on the same animal (eg.: pre- and post-test), a paired t-test was performed. Independent t-tests were performed to test for differences between treatments on two different groups of animals. Wilcoxon test was used as the non-parametric analog of the paired t-test (Zar, 1984). Correlation analysis were used to establish a relationship between pharmacokinetics and pharmacodynamics in each species at different doses after i.v. and s.c. administration. Statistical packages such as Systat® (Wilkinson, 1990) and Primer of Biostatistics (Glantz, 1992) were used for statistical analysis.

Approval of Protocols

All animal protocols were reviewed and approved by the following committees:

1. Institutional Animal Care and Use Committee (IACUC), Loyola University Chicago.

The IACUC approval numbers for the individual protocols are listed below:

i) In vivo antithrombotic activity, hemorrhagic and pharmacokinetic/ pharmacodynamic studies in rabbits after single i.v. and s.c. administration of rHV2: # 92-015.

ii) Pharmacokinetic/ pharmacodynamic, urinary excretion and renal function studies in rats after single i.v. and s.c. administration of rHV2: # 93-020-1a.

iii) Pharmacokinetic and pharmacodynamic studies in dogs after single and multiple i.v. and s.c. administration of rHV2: # 93-060.

2. Radiation Control Committee, Loyola University Chicago.

CHAPTER IV

RESULTS

In Vitro Studies

1. Construction of Concentration-Response Curves after *In Vitro* Supplementation of rHV2 in Plasma Systems

rHV2 was supplemented in normal rabbit plasma (NRP), normal rat plasma (NRatP), normal dog plasma (NDP) and blood bank plasma (BBP) to obtain plasma concentrations of 0 to 10 μg/mL. The supplemented plasma samples were screened for various coagulation tests and amidolytic anti-IIa activity, as described under the "Materials, Methods and Experimental Protocols" section. The supplementation of rHV2 and construction of concentration-response curves were performed on three different replicates using three separate pooled plasma preparations (sample size of at least six, each). Hence, each observation in the figures in this section represents the mean from three separate experiments ± SEM, and each individual experiment was performed in duplicate. The results from these assays are expressed in terms of adjusted concentration of rHV2 in the final assay system.

Concentration-response curves after rHV2 supplementation in NRP. The rHV2 supplemented NRP samples were initially analyzed using coagulation

tests: TT (10 U/mL), Ca^{+2}TT (10 U/mL), Ca^{+2}TT (20 U/mL) and Ca^{+2}TT (30 U/mL). The results from this experiment are depicted in Figure 12. The baseline values for TT (10 U/mL), Ca^{+2}TT (10 U/mL), Ca^{+2}TT (20 U/mL) and Ca^{+2}TT (30 U/mL) were 26.9 ± 3.1, 20.46 ± 2.45, 10.47 ± 1.02 and 6.5 ± 0.25 seconds (mean ± SEM), respectively. As the thrombin strength in the reagent was increased, the concentration-response curves shifted to the right, as indicated in Figure 12. The clotting time, as measured by TT (10 U/mL), exhibited a steep increase reaching ≥ 300 seconds at a low concentration of 0.9 pM/mL. However, when the thrombin reagent (10 U/mL) was reconstituted in $CaCl_2$ solution, a sigmoidal curve was obtained with at least 6 points in the linear portion of the curve. The clotting time, as measured by this assay {Ca^{+2}TT (10 U/mL)}, reached ≥ 300 seconds at rHV2 concentration of approximately 8 pM/mL.

On the other hand, when the calcium thrombin reagent was modified to 20 U/mL, a higher rHV2 concentration was required to exhibit measurable increases from baseline values, as compared to Ca^{+2}TT (10 U/mL). There were only 2 to 3 points in the linear portion with a sharp increase in Ca^{+2}TT (20 U/mL) to ≥ 300 seconds at rHV2 concentration of 10 pM/mL, as shown in Figure 12. An additional modification in the thrombin reagent to 30 U/mL exhibited very little concentration dependency, with almost no change in clotting times compared to baseline value (6.5 seconds) up to a concentration

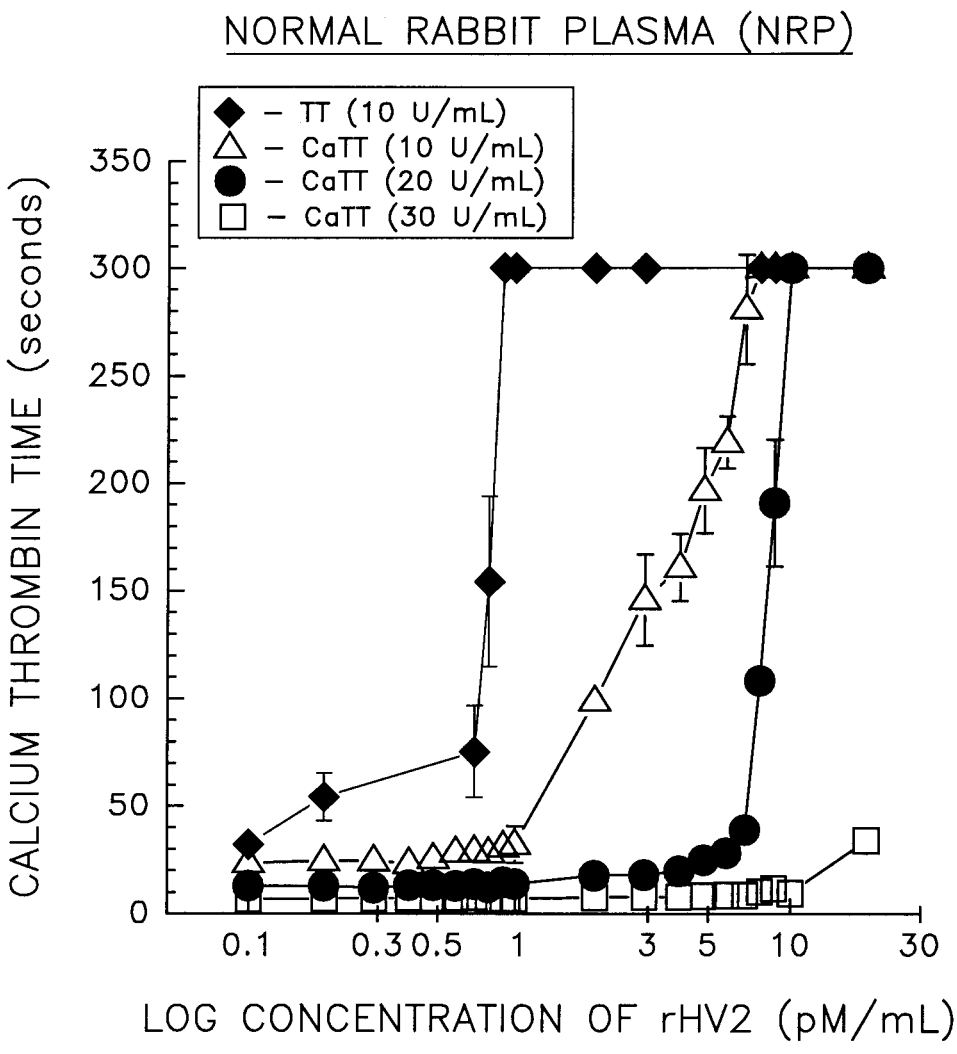


Figure 12. *In vitro* calibration curves using thrombin time and calcium thrombin time after supplementation of rHV2 in normal rabbit plasma. Symbols represent TT (10 U/mL): ◆, Ca^{+2}TT (10 U/mL): Δ, Ca^{+2}TT (20 U/mL): ● and Ca^{+2}TT (30 U/mL): □. The concentrations of rHV2 represent the final concentration of rHV2 corrected for volume in each assay system. All data represent the mean value from three individual experiments ± SEM, each performed in duplicate.

of 10 pM/mL. The results from this set of experiments led to the conclusion that Ca^{+2}TT (10 U/mL) would be the most optimal thrombin time, or more appropriately, modified thrombin time, that can used to monitor *in vitro* anticoagulant activity of rHV2.

The observed results with Ca^{+2}TT (10 U/mL) were compared with other *in vitro* assays, as illustrated in Figure 13. In this experiment, rHV2 supplemented NRP samples were analyzed using Ca^{+2}TT (10 U/mL), APTT (OT), APTT (Dade) and amidolytic anti-IIa assay. The baseline clotting times using Ca^{+2}TT (10 U/mL), APTT (OT) and APTT (Dade) were 20.46 ± 2.45, 57.05 ± 1.56 and 14.7 ± 4.2 (mean ± SEM), respectively, in NRP. Figure 13 illustrates a shift to the right in the concentration-response curves from Ca^{+2}TT (10 U/mL) to APTT (OT) and then to APTT (Dade). The thrombin inhibition curve using amidolytic anti-IIa assay was aligned between Ca^{+2}TT (10 U/mL) and APTT (OT). APTT (OT) was linear in the rHV2 concentration range between 10 and 150 pM/mL, beyond which the response was ≥ 300 seconds. APTT (Dade) exhibited very little dose dependency until about 150 pM/mL. The linear range for the chromogenic anti-IIa assay was between 1 and 10 pM/mL.

Because of the wide differences in the biochemical conditions, marked variations in the shape of concentration-response curves have been noted. The initiation of the observed anticoagulant/antithrombin effects was dependent on the type of assay used. It is clear from this comparison that the

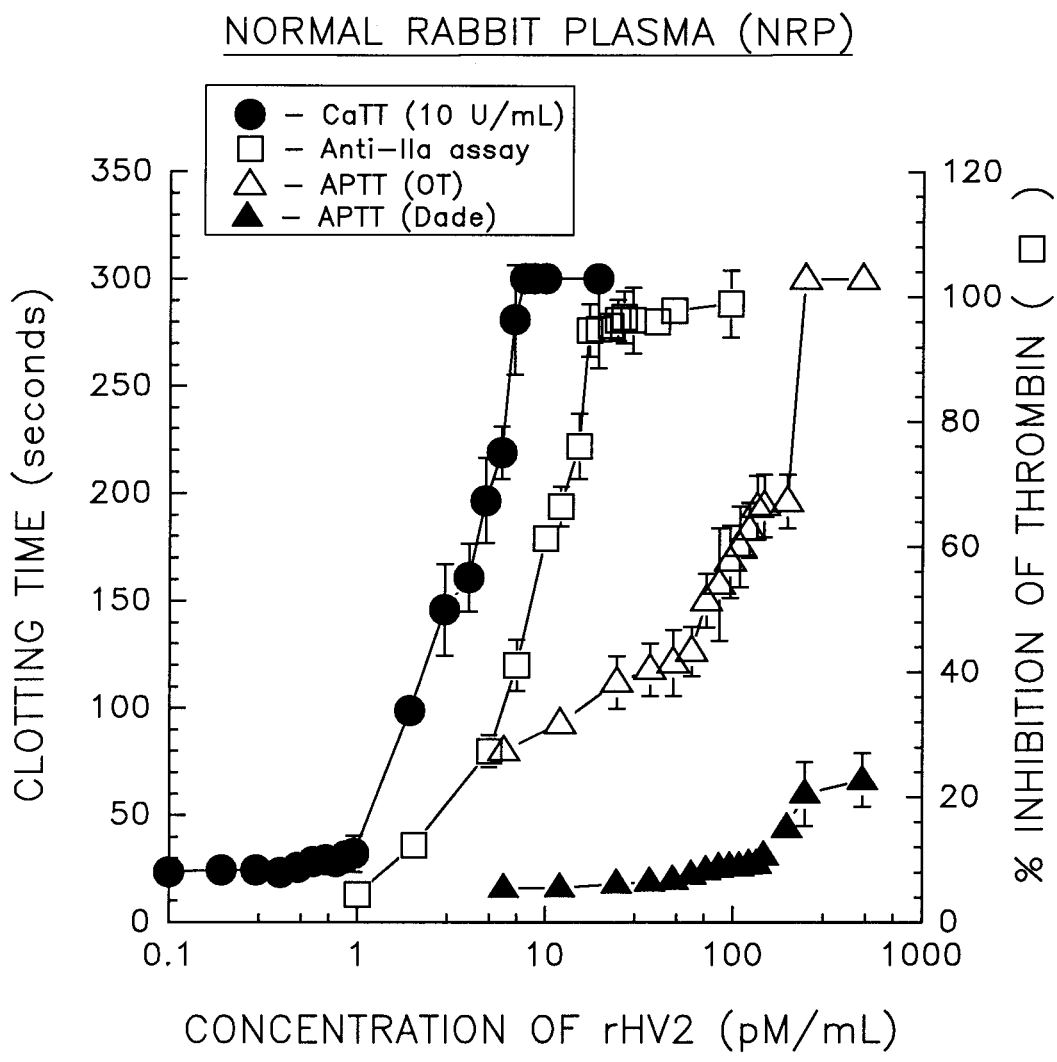


Figure 13. *In vitro* antithrombin profile of rHV2 supplemented in normal rabbit plasma (NRP). Symbols represent Ca^{+2}TT (10 U/mL): ● , APTT (OT): Δ , APTT (Dade): ▲, and chromogenic anti-IIa assay: □. The concentrations of rHV2 represent the final concentration of rHV2 corrected for volume in each assay system. All data are mean observations from three individual experiments, each performed in duplicate ± SEM.

relative sensitivity range of each of these assays for rH differs significantly. To better express these results, the slopes of the concentration-response curves were calculated in the selected linear range of each of the assays. The slopes were calculated using a least square regression analysis of the points in the linear portion of each curve (Miller and Freund, 1977). The slopes for the linear portions of the concentration-response curves in NRP, as well as in the other plasma preparations, are listed in Table 5.

In the clotting assays, a wide variation in the values of calculated slopes was observed in NRP samples (Table 5). When the Ca^{+2}TT (10 U/mL) slope was compared with APTT (OT) and APTT (Dade), marked differences were noted ($p<0.05$, one-way ANOVA followed by Tukey's multiple comparison test). Even within the two APTT reagents, an almost 6-fold difference ($p<0.05$) was observed in the slopes {0.65 seconds/pM/mL with APTT (OT) versus 0.1 second/pM/mL with APTT (Dade)}.

The correlation coefficients for the linear portion of concentration-response curves were found to be 0.99, 0.95, 0.86 and 0.98 for the Ca^{+2}TT (10 U/mL), APTT (OT), APTT (Dade) and chromogenic anti-IIa assay, respectively.

Concentration-response curves after rHV2 supplementation in NDP. rHV2 was supplemented in NDP in concentration ranges similar to that described above. Figure 14 shows the results from rHV2 supplemented NDP

samples that were analyzed using Ca^{+2}TT (10 U/mL), APTT (OT), APTT (Dade), and the anti-IIa assay. The baseline values for Ca^{+2}TT (10 U/mL), APTT (OT) and APTT (Dade) in NDP were 16.4 ± 2.05, 18.6 ± 3.2 and 13.5 ± 1.2 (mean ± SEM), respectively. The alignment of the concentration-response curves was similar to that found with NRP samples. The response curves for Ca^{+2}TT (10 U/mL), anti-IIa assay and APTT (OT) were almost parallel to each other with a shift to the right. The linear ranges of the three coagulation assays and the anti-IIa assay were similar to that obtained with NRP.

The correlation coefficients for the linear portions of the response curves with Ca^{+2}TT (10 U/mL), APTT (OT), APTT (Dade) and chromogenic anti-IIa assay in NDP were 0.92, 0.98, 0.99 and 0.96, respectively. The analysis of slopes obtained from the concentration-response curves revealed similar results to those obtained in the rabbit plasma studies. The results from the coagulation assays in NDP showed similar trends as in NRP, where major differences were noted between slopes of Ca^{+2}TT (10 U/mL) {40.31 seconds/pM/mL}, APTT (OT) {1.74 seconds/pM/mL) and APTT (Dade) {0.26 seconds/pM/mL}. The slope of the amidolytic anti-IIa assay response in NDP was nearly identical to that obtained in the rabbit plasma (4.53 % thrombin

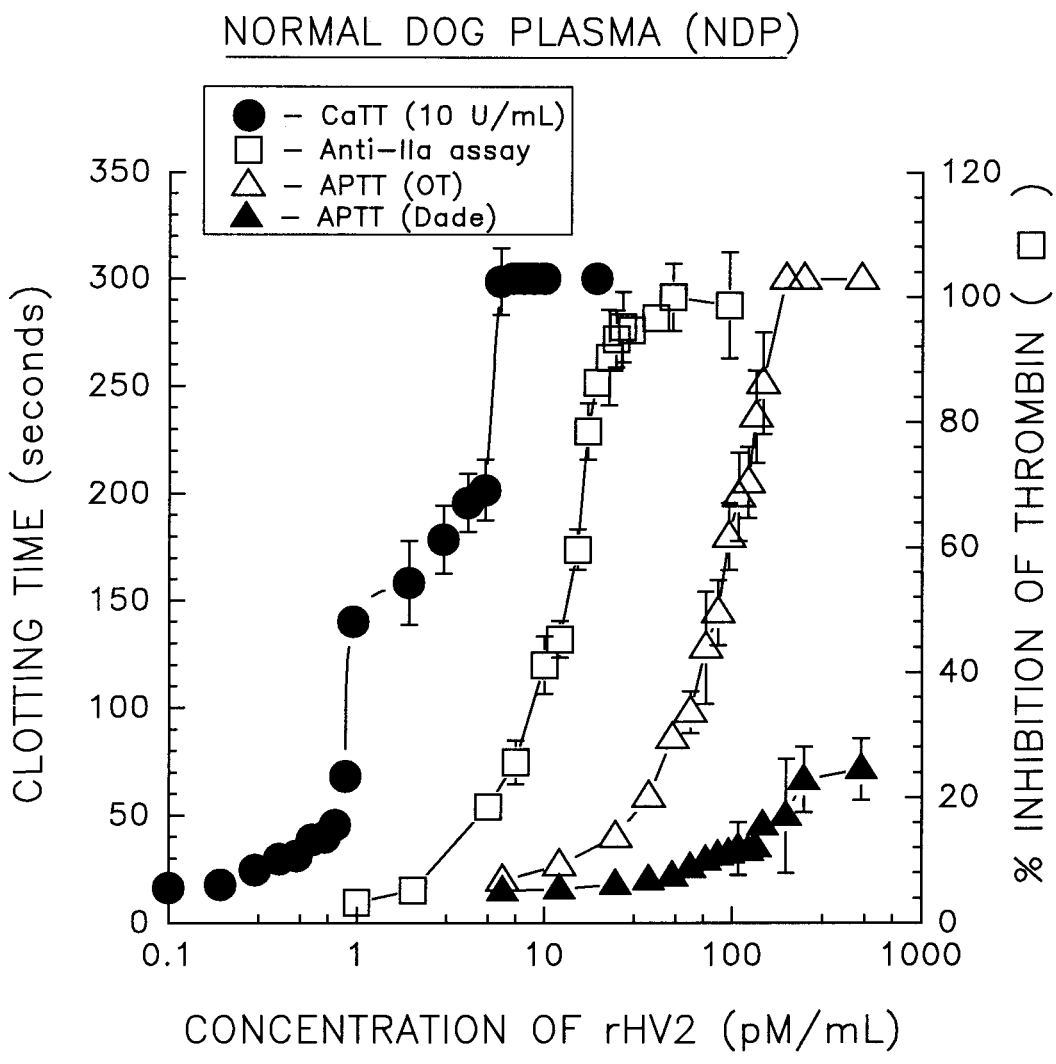


Figure 14. *In vitro* antithrombin profile of rHV2 supplemented in normal dog plasma (NDP). Symbols represent Ca^{+2}TT (10 U/mL): ● , APTT (OT): Δ , APTT (Dade): ▲, and chromogenic anti-IIa assay: □ . The concentrations of rHV2 represent the final concentration of rHV2 corrected for volume in each assay system. All data are mean observations from three individual experiments, each performed in duplicate ± SEM.

inhibition/pM/mL-NRP vs. 3.97 % thrombin inhibition/pM/mL-NDP, Table 5).

Concentration-Response curves after rHV2 supplementation in BBP. Figure 15 illustrates the concentration-response curves obtained after supplementation of rHV2 in BBP. The curves obtained with the three coagulation tests and amidolytic assay were similar to those obtained with NRP and NDP samples. The baseline values for Ca^{+2}TT (10 U/mL), APTT (OT) and APTT (Dade) were 18.9 ± 1.2, 27.56 ± 3.9 and 18.3 ± 2.6 (mean ± SEM), respectively. The corresponding correlation coefficients for the linear portions of the response curves were 0.94, 0.95, and 0.98, respectively. The correlation coefficient for the amidolytic anti-IIa assay was 0.97.

The results from the slope analysis of the concentration-response curves in blood bank plasma were similar to those obtained in rabbit and dog plasma (Table 5). The slope of Ca^{+2}TT (10 U/mL) was lower in BBP (20.74 seconds/pM/mL) compared with that in NRP (37.43 seconds/pM/mL) and NDP (40.31 seconds/pM/mL). However, this difference was not significant. The linear range for all of the assays in BBP was approximately the same as that found in NRP and NDP. The slope of APTT (Dade) in BBP (0.45 seconds/pM/mL) was significantly higher ($p<0.05$) than that found in NRP (0.1 second/pM/mL). However, it did not differ significantly from that obtained in NDP. Interestingly, the slope of the anti-IIa assay response in BBP was similar to those found with dog and rabbit plasma (Table 5).

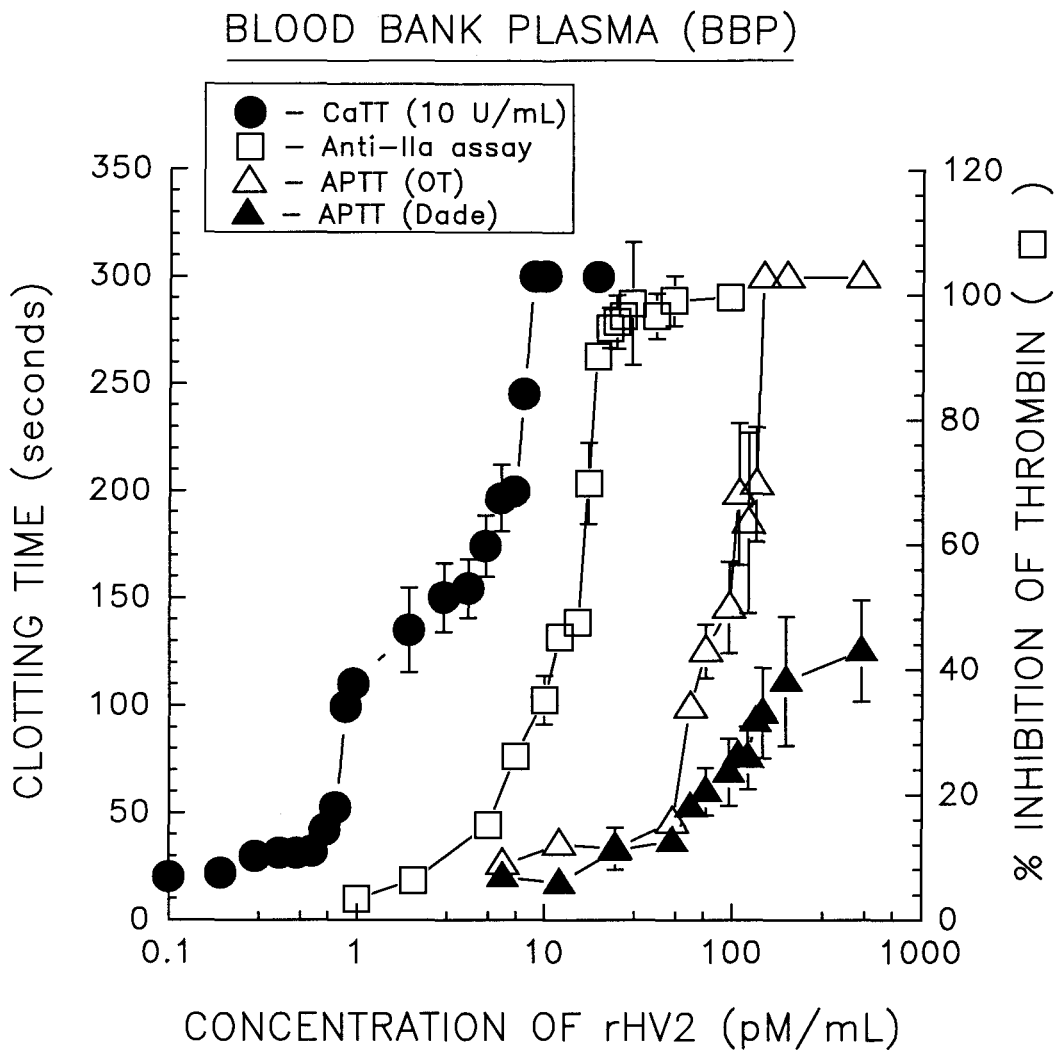


Figure 15. *In vitro* antithrombin profile of rHV2 supplemented in blood bank plasma (BBP). Symbols represent Ca^{+2}TT (10 U/mL): ● , APTT (OT): Δ , APTT (Dade): ▲, and chromogenic anti-IIa assay: □ . The concentrations of rHV2 represent the final concentration of rHV2 corrected for volume in each assay system. All data are mean observations from three individual experiments, each performed in duplicate ± SEM.

Concentration-response curve after rHV2 supplementation in NRatP.

Due to the limited amount of blood that can be drawn from rats, only the amidolytic anti-IIa assay was performed in rHV2 supplemented NRatP. The *in vitro* antithrombin activity measured by amidolytic anti-IIa assay after rHV2 supplementation in NRatP is shown in Figure 16. A higher rHV2 concentration was required to produce equivalent inhibition of thrombin compared with that observed in NRP, NDP and BBP. The linear range for this assay in NRatP was 10 to 100 pM/mL compared with 1 to 10 pM/mL in the other plasma preparations. A much lower value of slope (0.65 % thrombin inhibition/pM/mL) was obtained with rat plasma (Table 5).

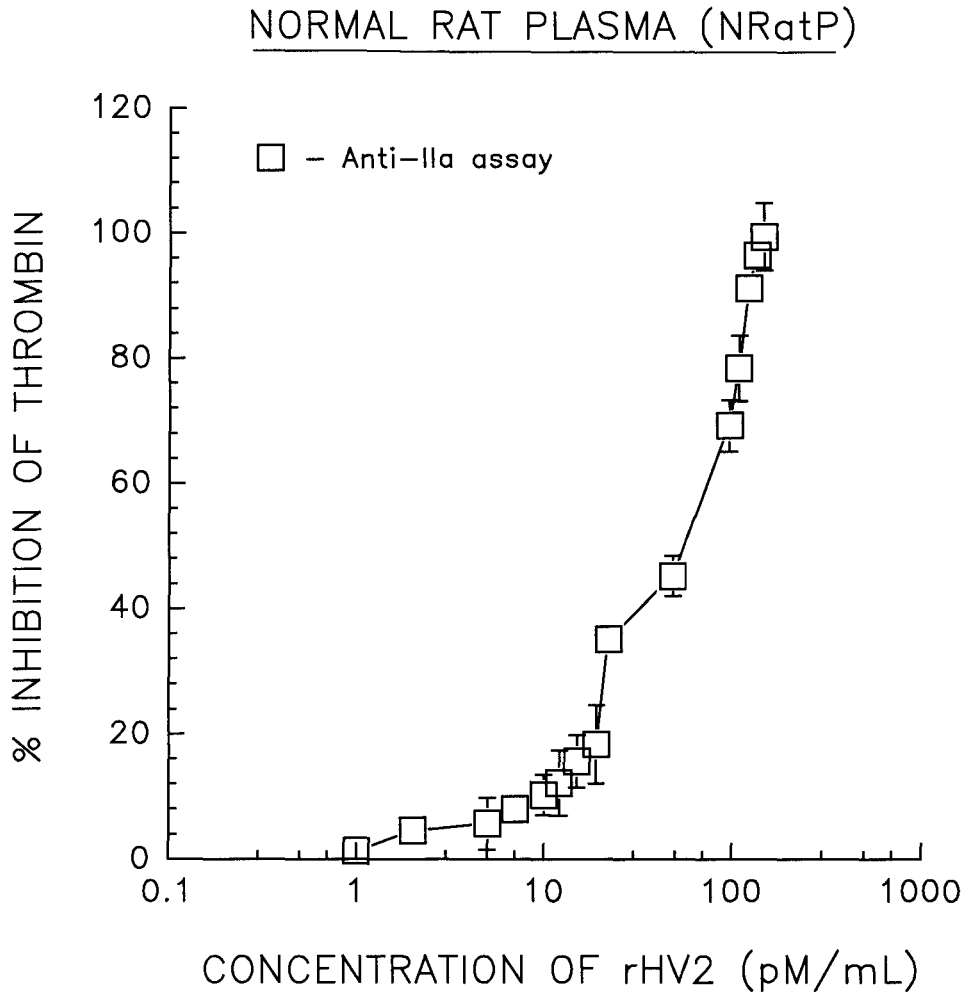


Figure 16. *In vitro* standard curve for chromogenic anti-IIa assay after supplementation of rHV2 in normal rat plasma (NRatP). The concentrations of rHV2 represent the final concentration of rHV2 corrected for volume in each assay system. All data represent the mean value from three individual experiments ± SEM, each performed in duplicate.

TABLE 5

SLOPES FROM CONCENTRATION-RESPONSE CURVES AFTER *IN VITRO* SUPPLEMENTATION OF rHV2 IN VARIOUS PLASMA SYSTEMS

Plasma System	Slope from Concentration-Response Curve (Mean ± SEM)			
	$Ca^{+2}TT$ (10 U/mL)	APTT (OT)	APTT (Dade)	Anti-IIa
NRP	37.43* ± 4.37	0.65** ± 0.11	0.10@ ± 0.07	4.53 ± 0.60
NDP	40.31* ± 11.82	1.74 ± 0.07	0.26 ± 0.08	3.97 ± 0.53
BBP	20.74* ± 4.40	1.73 ± 0.46	0.45 ± 0.09	4.79 ± 0.84
NRatP	---	---	---	0.65# ± 0.06
Units	sec/pM/mL	sec/pM/mL	sec/pM/mL	%Inh/pM/mL

* - $p<0.05$, $Ca^{+2}TT$ (10 U/mL) versus APTT (OT) and APTT (Dade) in NRP, NDP and BBP
** - $p<0.05$, APTT (OT) versus APTT (Dade) in NRP
@ - $p<0.05$, APTT (Dade), NRP versus BBP
\# - $p<0.05$, Anti-IIa assay, NRatP versus NRP, NDP and BBP
(one-way ANOVA followed by Tukey test).

2. Determination of Specific Activity of rHV2 using a Thrombin Titration Method

Figure 17 illustrates the residual thrombin activity obtained after addition of increasing concentrations of rHV2, supplemented in normal saline. Thrombin activity was expressed as a percentage of the activity obtained in

control (saline) samples. The amidolytic activity of thrombin on its substrate (Spectrozyme® TH) decreased progressively in the presence of increasing amounts of inhibitor (rHV2) until it reached a steady level of 5 to 10% residual thrombin activity. Figure 17 indicates that approximately 63.5 ng/mL final concentration of rHV2 completely neutralized one NIH unit of α-thrombin. This meant that the specific activity of this batch of rHV2, as measured by thrombin titration method, was 15,873.02 ATU/mg protein. Table 6 lists the specific activities of rHV2 calculated by above method from three separate determinations (each performed in duplicate). The average specific activities of rHV2 obtained from the three individual experiments was 15,877.67 ATU/mg protein, which closely matched the calculated potency from the average thrombin titration curve depicted in Figure 17. This specific activity was approximately the same as the manufacturer's assigned potency of rHV2 (14,495 ATU/mg).

TABLE 6

DETERMINATION OF SPECIFIC ACTIVITY (ATU/mg) of rHV2 USING THROMBIN TITRATION METHOD

Experiment No.	Specific Activity
1	16,260.16
2	15,748.00
3	15,625.00
Mean specific activity ± S.D.	15,877.67 ± 336.77
Specific activity from average curve	15,873.02

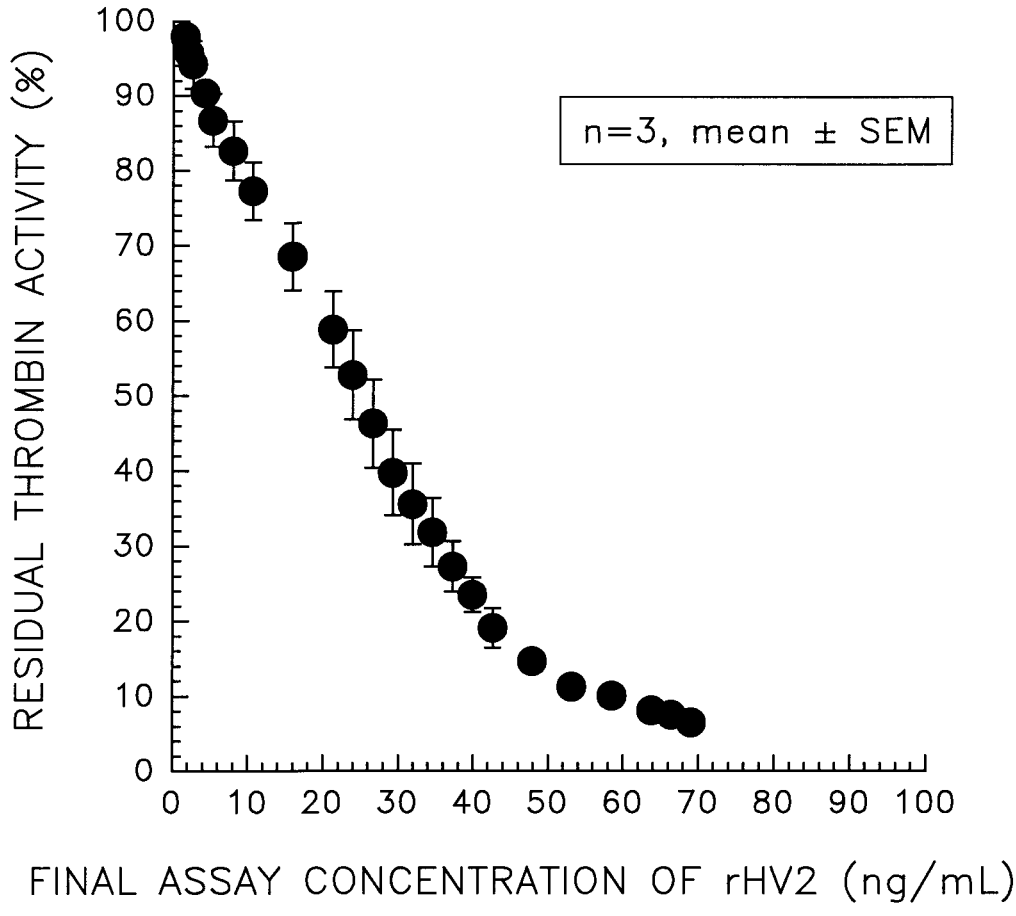


Figure 17. Thrombin titration curve for determination of specific activity of rHV2. Each data point represents the mean of three individual experiments, each determined in duplicate ± SEM.

3. Determination of Protein Content of rHV2 using Δ 215-225 Method

The difference in mean optical densities (n = 3) at 215 and 225 nm (Δ 215-225 nm) of rHV2 solutions at concentrations of 25, 50 and 100 μg/mL was found to be 0.19 ± 0.06, 0.39 ± 0.03 and 0.71 ± 0.1 (mean ± SEM), respectively. This translated to a protein content of 27.4, 56.2 and 102.2 μg/mL of protein in these solutions, respectively.

In Vivo Studies

1. Assessment of Antithrombotic Profile of rHV2 using a Modified Jugular Vein Stasis Thrombosis Model in Rabbits - A Dose-Ranging Study

The antithrombotic properties of rHV2 were evaluated using a modified rabbit jugular vein stasis thrombosis model, as described in the chapter on "Materials, Methods and Experimental Protocols". rHV2 was used in i.v. doses of 6.25, 12.5 and 25 μg/kg and s.c. doses of 125, 250 and 375 μg/kg. Appropriate saline treated control groups (n = 5) were included for each route of administration. The results obtained from these studies are described in the following sections.

I.V. administration. I.V. administration of rHV2 exhibited a dose dependent antithrombotic activity with increasing doses, as illustrated in Figure 18. Saline treated, control rabbits exhibited clot scores of +3 and +4 after 10 minute and 20 minute stasis times, respectively. With rHV2 administration, the antithrombotic activity increased progressively until clot

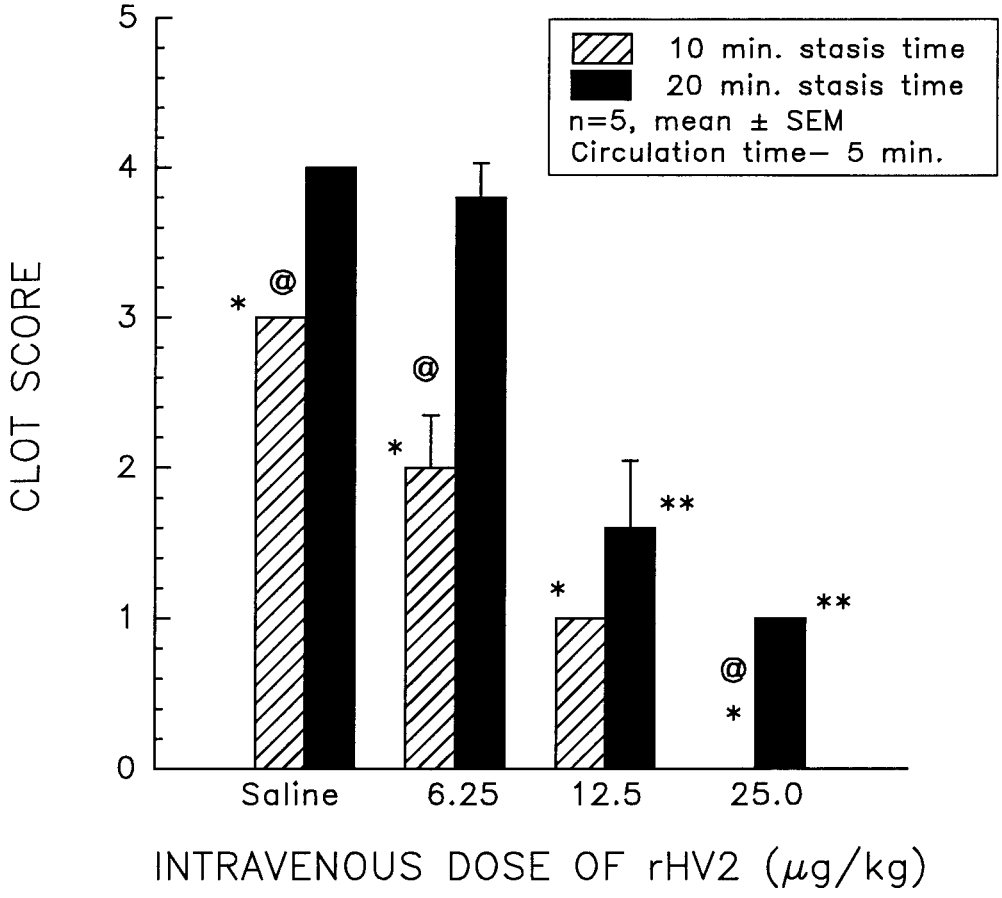


Figure 18. *In vivo* antithrombotic activity of rHV2 after i.v. administration in rabbits. Increasing doses of 0 (saline), 6.25, 12.5 and 25 μg/kg were administered in male, New Zealand White rabbits (n=5 per dose), in the modified jugular vein stasis thrombosis model. rHV2 circulation time was 5 minutes followed by a 10 and 20 minute stasis in the left and right sides, respectively. All data represent a mean of 5 clot scores ± SEM.

* - significant variation ($p<0.05$) in clot scores between all doses including saline control after 10 minute stasis time, and ** - significant difference ($p<0.05$) in clot scores between 12.5 and 25 μg/kg rHV2 treatments with corresponding saline and 6.25 μg/kg rHV2 treatment after 20 minute stasis time (Kruskal-Wallis one-way ANOVA followed by Mann-Whitney test). @ - significant increase in clot scores after specific doses of rHV2 at 20 minutes versus 10 minutes (Wilcoxon test).

scores of 0 and +1 were obtained at 10 minute and 20 minute stasis times, respectively, at a dose of 25 μg/kg of rHV2. At the 10 minute stasis time, clot scores obtained after all rHV2 doses and saline treatment were significantly different from each other ($p < 0.05$, Kruskal-Wallis non-parametric one-way ANOVA, followed by multiple comparison using Mann-Whitney test). At the 20 minute stasis time, however, there was no significant variation in the antithrombotic responses obtained after saline treatment and the lowest dose i.e. 6.25 μg/kg of rHV2. Significant increases in antithrombotic activity ($p < 0.05$) were seen at the higher doses, i.e. 12.5 and 25 μg/kg of rHV2, when clot scores were compared to those obtained after saline and 6.25 μg/kg rHV2 treatment (Figure 18).

Increasing the stasis time to 20 minutes on one jugular vein segment significantly decreased the antithrombotic activity of rHV2 when compared to corresponding results after 10 minute stasis. This was observed after treatment with all doses of rHV2 except 12.5 μg/kg ($p < 0.05$, Wilcoxon test). Complete antithrombotic activity was observed with a dose of 25 μg/kg, i.v., indicated by a clot score of 0 after a stasis time of 10 minutes.

The results from global coagulation assays after i.v. administration of rHV2 in rabbits are depicted in Figures 19 to 22. In the case of APTT (OT), there was no variation in responses between the three doses of rHV2 and saline treatment both at 5 (post-drug) and 11 minutes (post-FEIBA®) after rHV2 administration, as shown in Figure 19. As expected, the APTT (OT)

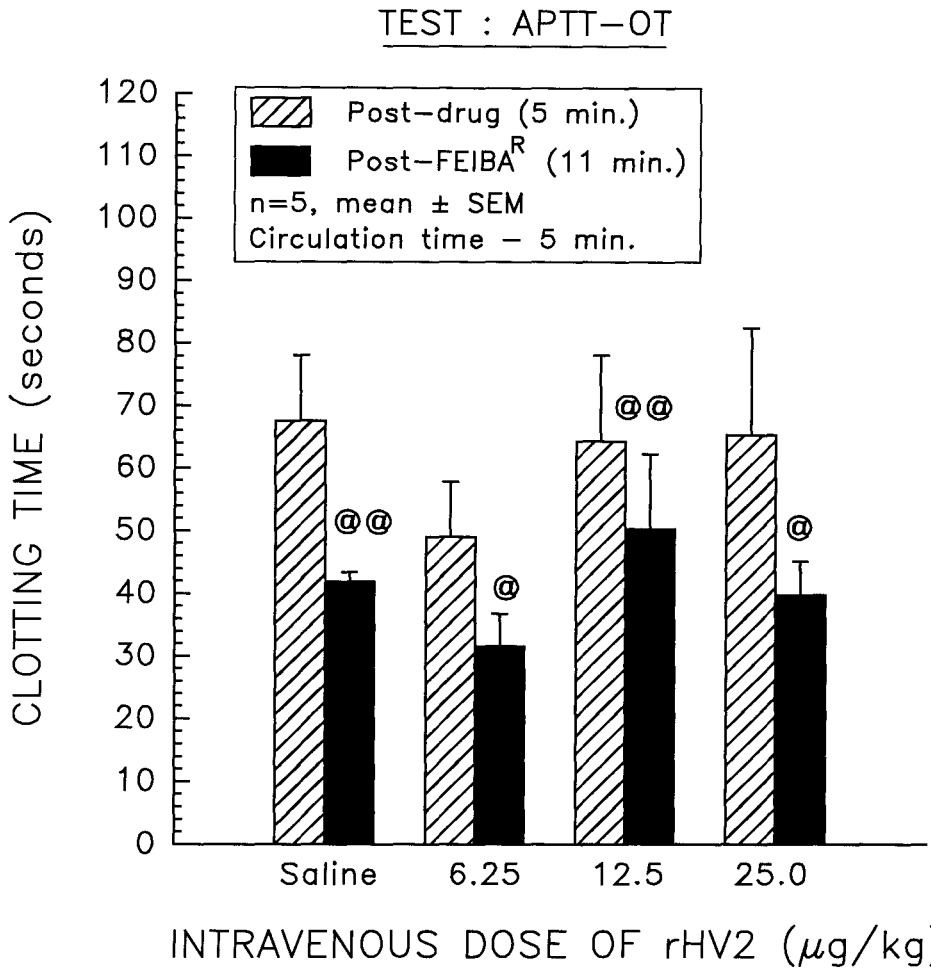


Figure 19. *Ex vivo* antithrombin activity of rHV2 using APTT (OT), after i.v. administration in rabbits. rHV2 was used in doses of 0, 6.25, 12.5 and 25.0 μg/kg in male, New Zealand White rabbits (n = 5). All data represent a mean of 5 responses each determined in duplicate ± SEM.

No significant difference between different treatment groups in APTT (OT) values in post-drug and post-FEIBA® samples (one-way ANOVA). @ and @@- significant difference ($p < 0.05$ and 0.1, respectively) in APTT (OT) values between post-drug and post-FEIBA® samples after administration of specific doses of rHV2 (paired t-test).

values after FEIBA® administration were significantly lower than post-drug APTT (OT) values after rHV2 administration at different doses ($p<0.1$ after saline and 12.5 μg/kg rHV2 and $p<0.05$ after 6.25 and 25 μg/kg rHV2, paired t-test).

In the case of APTT (Dade), significant increases ($p<0.05$) in clotting times were seen after the administration of 12.5 and 25 μg/kg rHV2 doses when compared with saline and 6.25 μg/kg rHV2 treatment, in both the post-drug and post-FEIBA® samples, as indicated in Figure 20. There was a significant decrease in APTT (Dade) after FEIBA® administration in the 25 μg/kg rHV2 treatment group (paired t-test).

The results obtained with TT (10 U/mL) are illustrated in Figure 21. TT (10 U/mL) showed a marked increase in clotting times ($\geq$ 300 seconds) after administration of 12.5 and 25 μg/kg of rHV2 ($p<0.05$ compared with corresponding saline and 6.25 μg/kg rHV2 treatment, one-way ANOVA followed by Tukey test). Administration of FEIBA® did not decrease the TT (10 U/mL) values significantly after administration of all doses of rHV2 (paired t-test, Figure 21). On the other hand, Ca^{+2}TT (10 U/mL) showed a more linear increase in response with increasing i.v. doses of rHV2, as shown in Figure 22. A significant increase ($p<0.05$, one-way ANOVA) was seen in Ca^{+2}TT (10 U/mL) in post-drug and post-FEIBA® samples when compared with corresponding saline and 6.25 μg/kg rHV2 treatment. There were significant decreases ($p<0.05$, paired t-test) in clotting times using this assay

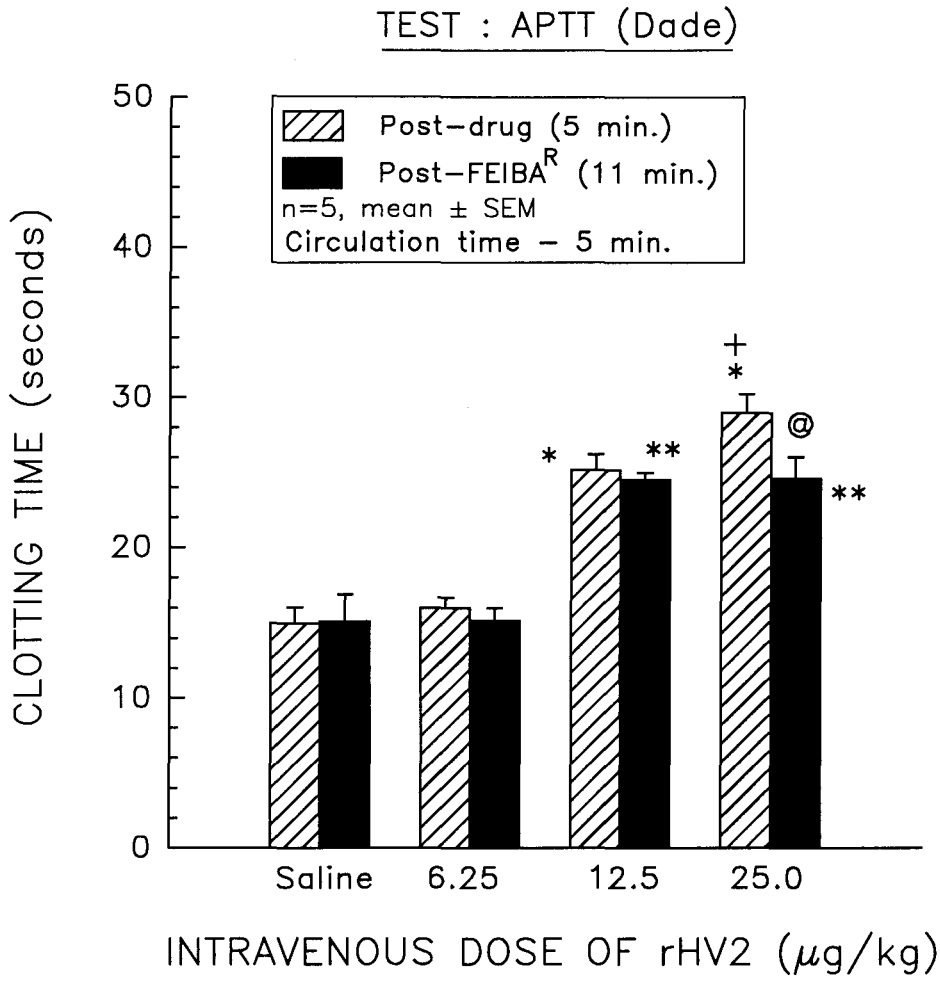


Figure 20. *Ex vivo* antithrombin activity of rHV2 using APTT (Dade) after i.v. administration in rabbits. rHV2 was used in doses of 0, 6.25, 12.5 and 25.0 μg/kg in male, New Zealand White rabbits (n = 5). All data represent a mean of 5 responses each determined in duplicate ± SEM.

* - significant difference ($p < 0.05$) in APTT (Dade) values from saline and 6.25 μg/kg rHV2 treated groups in post-drug samples, + - significant difference ($p < 0.05$) from 12.5 μg/kg rHV2 treated group in post-drug samples and ** - significant difference ($p < 0.05$) from saline and 6.25 μg/kg treated groups in post-FEIBA® samples (one-way ANOVA followed by Tukey test). @ - significant difference ($p < 0.05$) in APTT (Dade) between post-drug and post-FEIBA® samples after administration of 25 μg/kg of rHV2 (paired t-test).

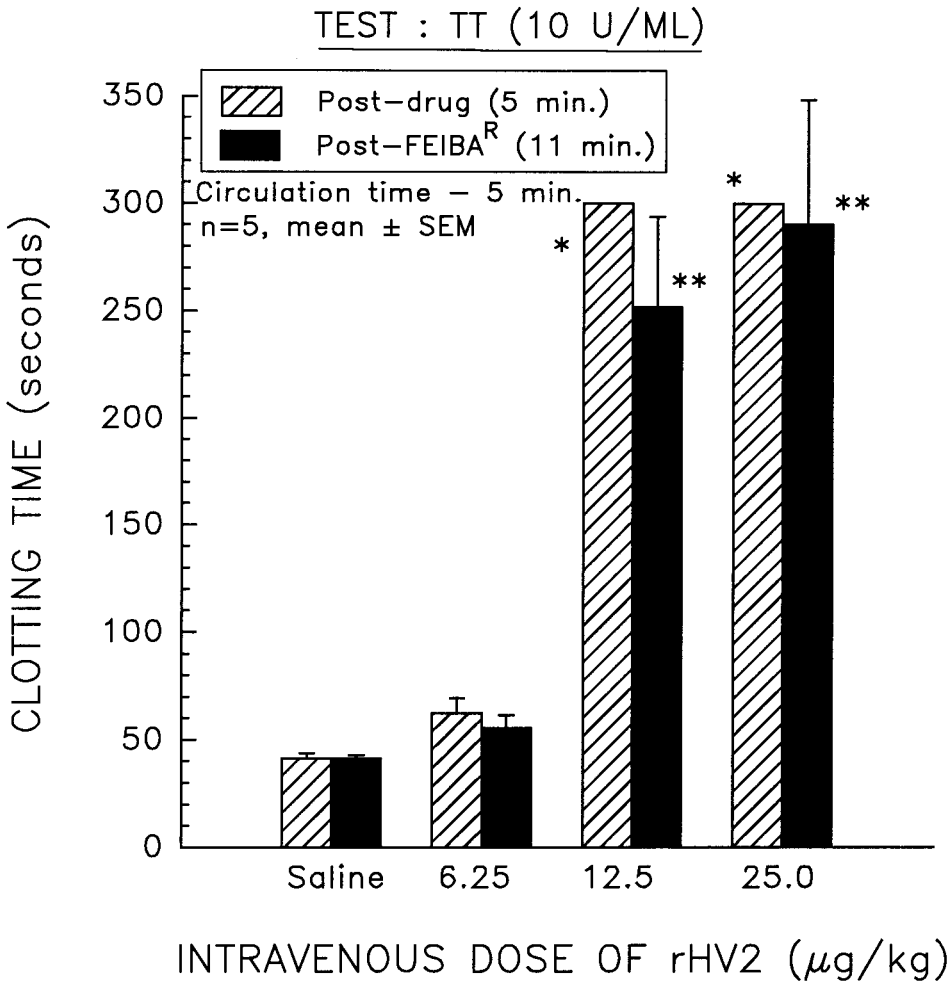


Figure 21. *Ex vivo* antithrombin activity of rHV2 using TT (10 U/mL), after i.v. administration in rabbits. rHV2 was used in doses of 0, 6.25, 12.5 and 25.0 μg/kg in male, New Zealand White rabbits (n = 5). All data represent a mean of 5 responses each determined in duplicate ± SEM.

* - significant difference ($p < 0.05$) in TT (10 U/mL) values compared to saline and 6.25 μg/kg rHV2 treated groups in post-drug samples and ** - significant difference ($p < 0.05$) from saline and 6.25 μg/kg treated groups in post-FEIBA® samples (one-way ANOVA followed by Tukey test).

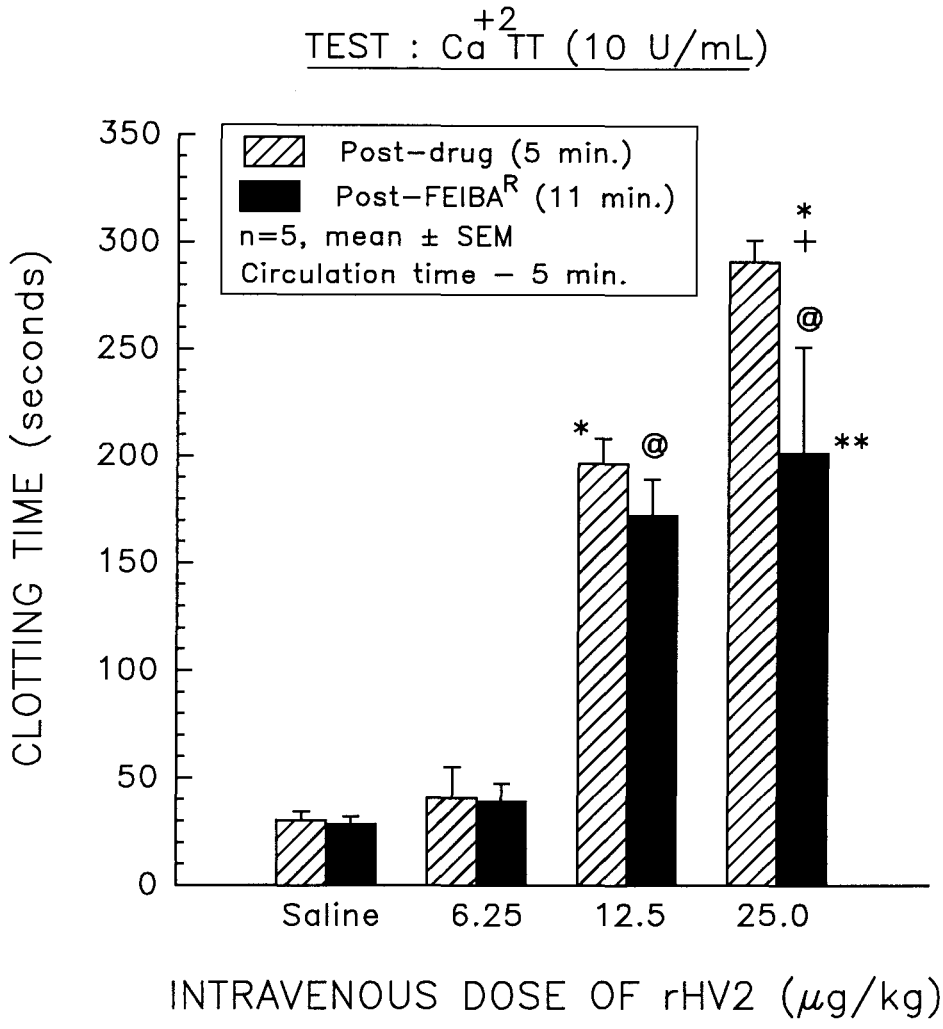


Figure 22. *Ex vivo* antithrombin activity of rHV2 using $Ca^{+2}TT$ (10 U/mL), after i.v. administration in rabbits. rHV2 was used in doses of 0, 6.25, 12.5 and 25.0 μg/kg in male, New Zealand White rabbits (n = 5). All data represent a mean of 5 responses each determined in duplicate ± SEM.

* - significant difference ($p < 0.05$) in $Ca^{+2}TT$ (10 U/mL) values from saline and 6.25 μg/kg rHV2 treated groups in post-drug samples, + - significant difference ($p < 0.05$) from 12.5 μg/kg rHV2 treated group in post-drug samples and ** - significant difference ($p < 0.05$) from saline, 6.25 and 12.5 μg/kg treated groups in post-FEIBA® samples (one-way ANOVA followed by Tukey test). @ - significant difference ($p < 0.05$) in $Ca^{+2}TT$ (10 U/mL) between post-drug and post-FEIBA® samples at specific doses of rHV2 (paired t-test).

after FEIBA® administration in the samples from the higher dose treatments (12.5 and 25 μg/kg). rHV2 administration at 25 μg/kg showed $Ca^{+2}TT$ (10 U/mL) values greater than or equal to 300 seconds (Figure 22).

Ex vivo antithrombin activity measured using the chromogenic antithrombin assay was expressed as "percent inhibition of thrombin" when compared with the thrombin inhibition produced by baseline samples from each corresponding rabbit, as illustrated in Figure 23. A dose dependent, linear increase in thrombin inhibition was observed with increasing i.v. doses of rHV2. A significant prolongation ($p < 0.05$, one-way ANOVA followed by Tukey test) of the thrombin inhibitory activity was seen after administration of increasing doses of rHV2. FEIBA® administration produced a significant decrease ($p < 0.05$, paired t-test) in anti-IIa activity in groups administered with the higher two doses of rHV2, as shown in Figure 23. The 25 μg/kg dose produced a thrombin inhibitory activity of almost 76 ± 6 %.

Selection of appropriate i.v. dose for time course study. From the *in vivo* clot score results and *ex vivo* clotting and anti-IIa results illustrated above, an i.v. dose of 25 μg/kg was chosen for further time course studies in rabbits using stasis thrombosis model. These results are described in the following sections.

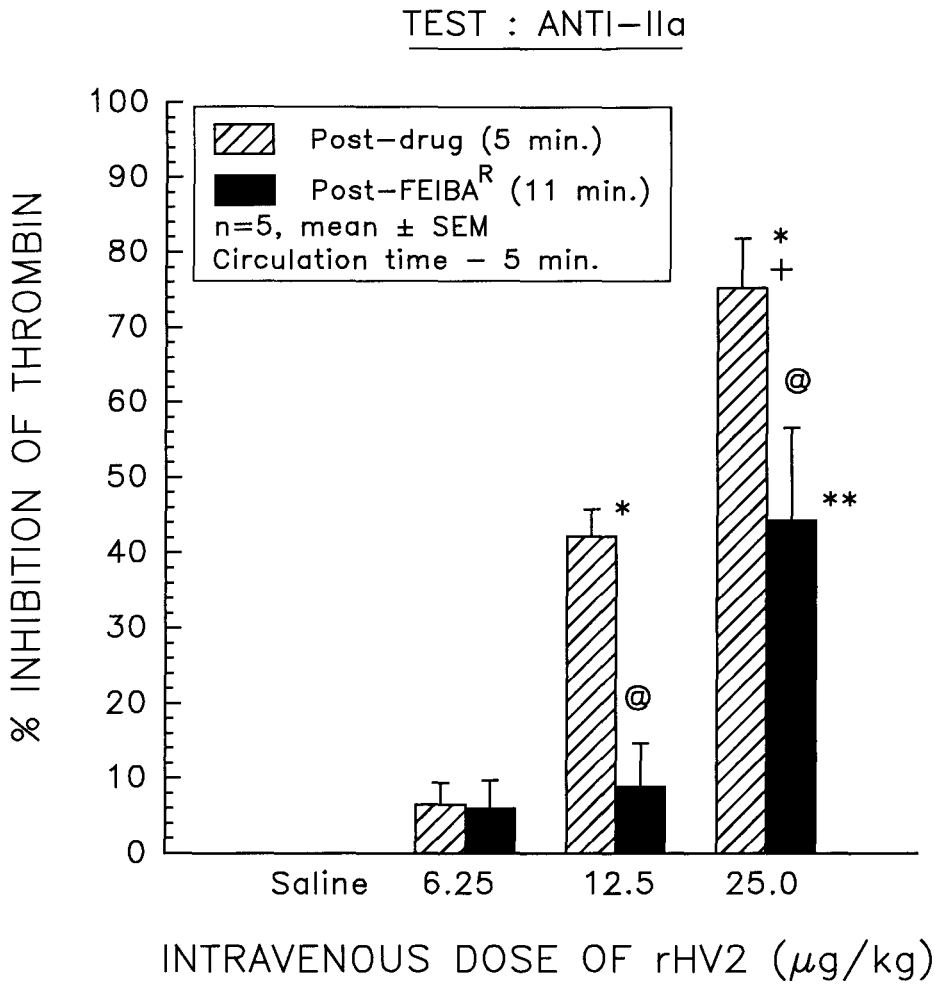


Figure 23. *Ex vivo* antithrombin activity of rHV2 using chromogenic anti-IIa assay, after i.v. administration in rabbits. rHV2 was used in doses of 0, 6.25, 12.5 and 25.0 μg/kg in male, New Zealand White rabbits (n=5). All data represent a mean of 5 responses each determined in duplicate ± SEM.

* - significant difference ($p<0.05$) in anti-IIa activity from saline and 6.25 μg/kg rHV2 treated groups in post-drug samples, + - significant difference ($p<0.05$) from 12.5 μg/kg rHV2 treated group in post-drug samples and ** - significant difference ($p<0.05$) from saline, 6.25 and 12.5 μg/kg treated groups in post-FEIBA® samples (one-way ANOVA followed by Tukey test). @ - significant difference ($p<0.05$) in antithrombin activity between post-drug and post-FEIBA® samples at specific doses of rHV2 (paired t-test).

S.C. administration. A dose dependent increase in antithrombotic activity was seen after s.c. administration of rHV2 using the modified jugular vein stasis thrombosis model. These results are in Figure 24. Saline treated groups exhibited clot scores of +3 and +4 after 10 minute and 20 minute stasis, respectively. Significantly stronger antithrombotic responses were obtained after administration of 250 and 375 μg/kg doses of rHV2 at 10 minute stasis time (clot scores = 0, $p < 0.05$, Kruskal-Wallis one-way ANOVA followed by Mann-Whitney test).

After 20 minute stasis, clot scores obtained with all doses of rHV2, including saline treatment, showed significant differences from each other ($p < 0.05$, Kruskal-Wallis one-way ANOVA followed by Mann-Whitney test). Using the Wilcoxon test, a significant decrease ($p < 0.05$) in antithrombotic activity was seen at 20 minutes when compared to that observed at 10 minute stasis time after s.c. administration of specific doses of rHV2 (Figure 24). A strong antithrombotic effect was observed after s.c. administration of rHV2 at a dose of 375 μg/kg as shown by mean clot scores of 0 and <1 at 10 minute and 20 minute stasis, respectively.

The results from the global coagulation assays after s.c. administration of rHV2 are shown in Figures 25 to 28. All three doses of rHV2, and FEIBA® administration showed a significant increase ($p < 0.05$, one-way ANOVA followed by Tukey test) in APTT (OT) values compared with corresponding

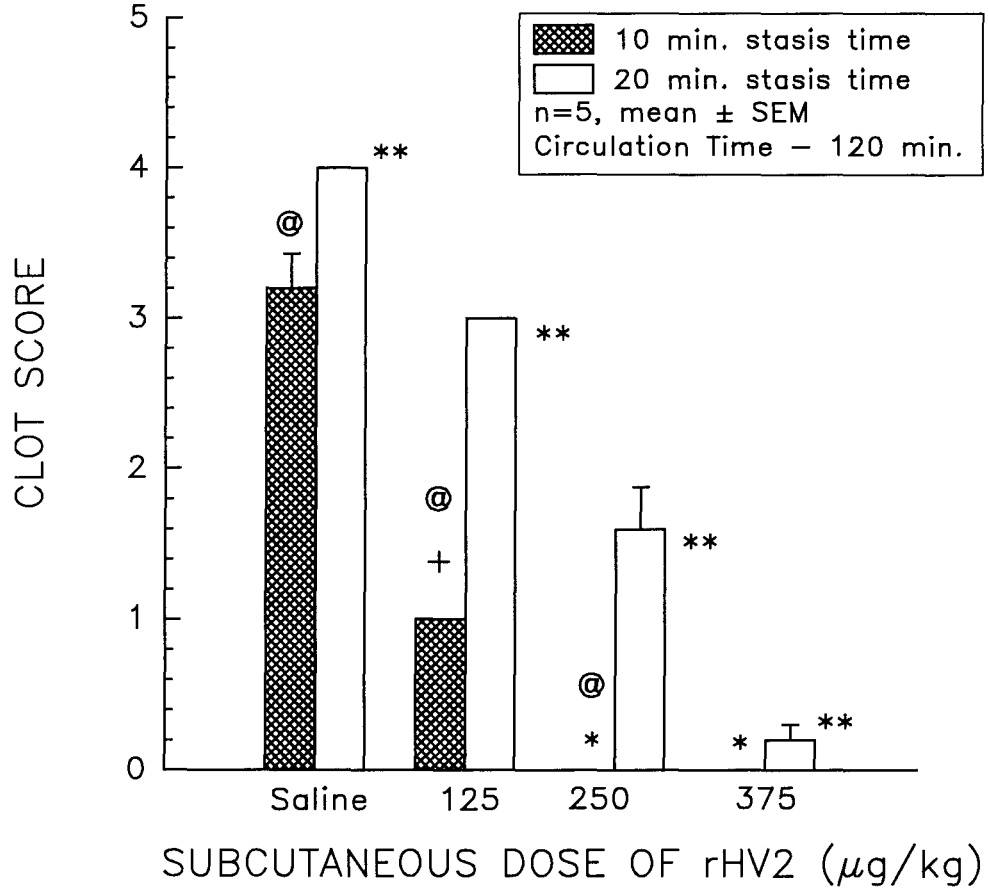


Figure 24. *In vivo* antithrombotic activity of rHV2 after s.c. administration in rabbits. rHV2 was used in increasing doses of 0 (saline), 125, 250 and 375 μg/kg in male, New Zealand White rabbits (n = 5 per dose), in the modified jugular vein stasis thrombosis model. rHV2 circulation time was 120 minutes followed by a 10 and 20 minute stasis in the left and right sides, respectively. All data represent a mean of 5 clot scores ± SEM.

* - significant difference ($p < 0.05$) in clot scores between 250 and 375 μg/kg rHV2 treatments and corresponding saline and 125 μg/kg treatments after 10 minute stasis time, + - significant difference in clot scores between saline and 125 μg/kg rHV2 treatment after 10 minute stasis time, and ** - significant variation ($p < 0.05$) in clot scores between all doses including saline control after 20 minute stasis time (Kruskal-Wallis one-way ANOVA followed by Mann-Whitney test). @ - significant increase in clot scores after specific doses of rHV2 (Wilcoxon test).

values after saline treatment, as shown in Figure 25. There was a significant decrease ($p<0.05$, paired t-test) in APTT (OT) values after administration of FEIBA® following the three rHV2 doses. On the other hand, there were no significant changes in clotting times, as measured by APTT (Dade), between the three s.c. doses of rHV2 and saline treatment, as illustrated in Figure 26 (one-way ANOVA followed by Tukey test). A weaker, yet significant, increase in APTT (Dade) was found only after administration of FEIBA® following 375 μg/kg rHV2 treatment, when compared with corresponding saline control.

TT (10 U/mL) showed a major increase in clotting times after administration of all three doses of rHV2, as indicated in Figure 27. Significant prolongation in TT (10 U/mL) was found after administration of all three s.c. doses of rHV2 and FEIBA® compared to corresponding saline treatment (one-way ANOVA followed by Tukey test). In contrast to i.v. administration, a linear response was not seen with increasing s.c. doses, as monitored by Ca^{+2}TT (10 U/mL) and indicated in Figure 28. A stronger increase was seen in clotting times after administration of rHV2 in all doses and FEIBA® compared with saline controls (one-way ANOVA followed by Tukey test).

The amidolytic antithrombin assay results showed a dose dependent linear increase in *ex vivo* activity after increasing s.c. administration of rHV2, as depicted in Figure 29. Significant increases in thrombin inhibitory response was seen after rHV2 administration and saline treatments as well as between

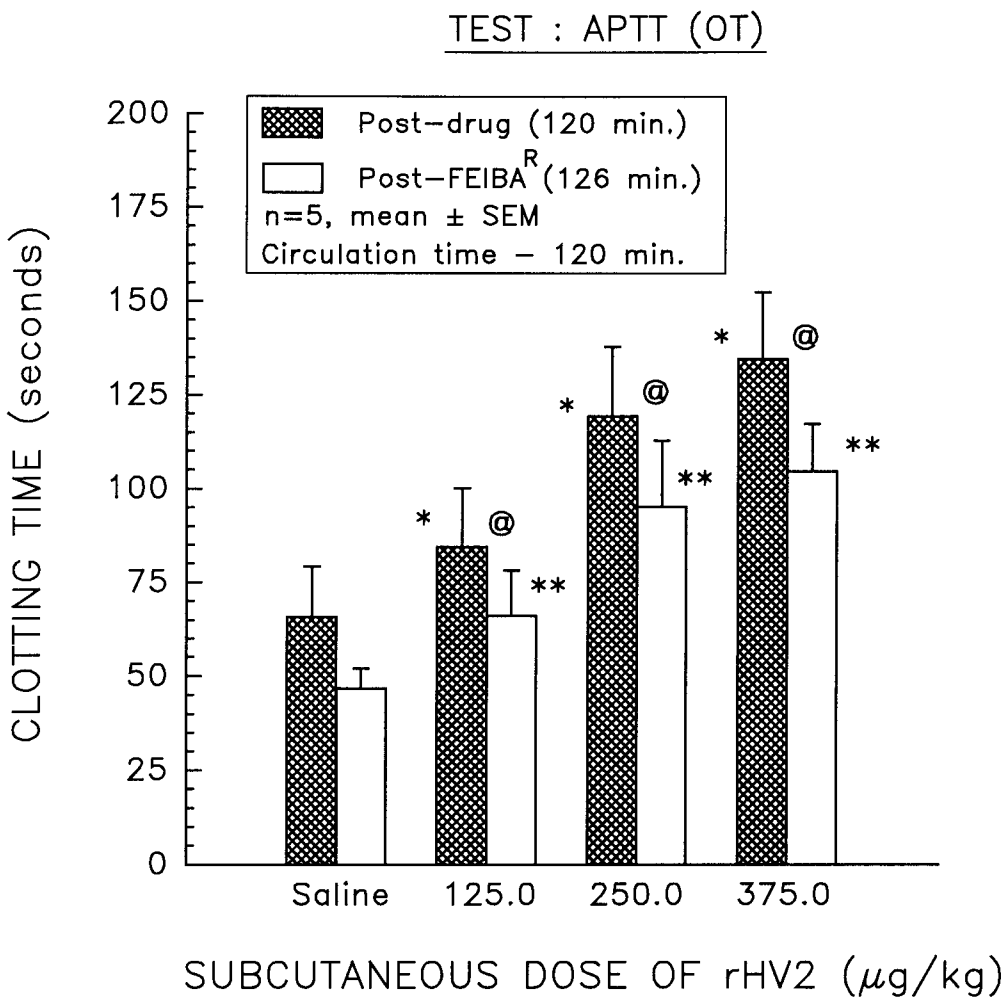


Figure 25. *Ex vivo* antithrombin activity of rHV2 using APTT (OT), after s.c. administration in rabbits. rHV2 was used in doses of 0, 125, 250 and 375 μg/kg in male, New Zealand White rabbits (n = 5). All data represent a mean of 5 responses each determined in duplicate ± SEM.

*, **- significant difference ($p < 0.05$) in APTT (OT) values compared to saline in post-drug and post-FEIBA® samples, respectively (one-way ANOVA followed by Tukey test). @-significant difference ($p < 0.05$) in APTT (OT) between post-drug and post-FEIBA® samples after administration of specific doses of rHV2 (paired t-test).

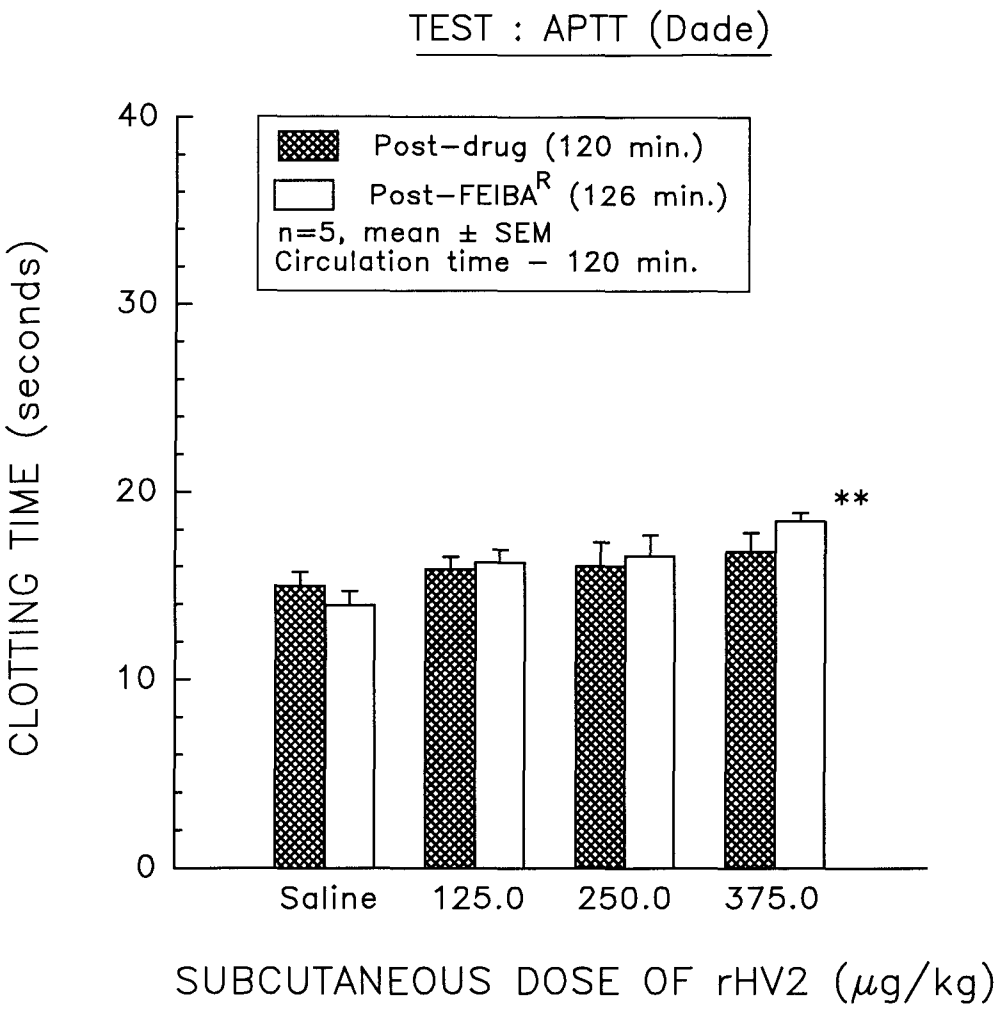


Figure 26. *Ex vivo* antithrombin activity of rHV2 using APTT (Dade), after s.c. administration in rabbits. rHV2 was used in doses of 0, 125, 250 and 375 μg/kg in male, New Zealand White rabbits (n = 5). All data represent a mean of 5 responses each determined in duplicate ± SEM.

**- significant difference ($p < 0.05$) in APTT (Dade) values compared to saline in post-FEIBA® samples (one-way ANOVA followed by Tukey test).

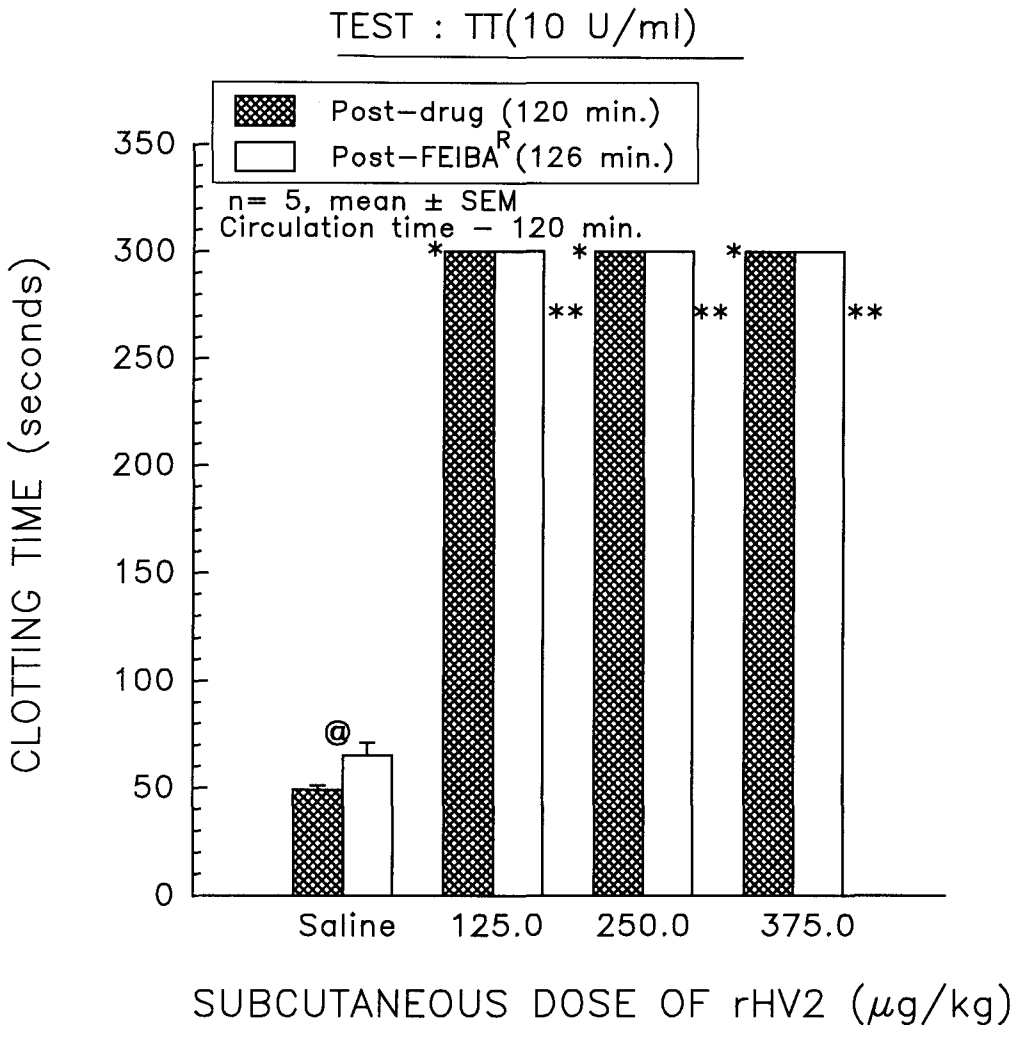


Figure 27. *Ex vivo* antithrombin activity of rHV2 using TT (10 U/mL), after s.c. administration in rabbits. rHV2 was used in doses of 0, 125, 250 and 375 μg/kg in male, New Zealand White rabbits (n = 5). All data represent a mean of 5 responses each determined in duplicate ± SEM.

*, **- significant difference ($p < 0.05$) in TT (10 U/mL) values compared to saline in post-drug and post-FEIBA® samples, respectively (one-way ANOVA followed by Tukey test). @-significant difference ($p < 0.05$) in TT (10 U/mL) between post-drug and post-FEIBA® samples after saline treatment (paired t-test).

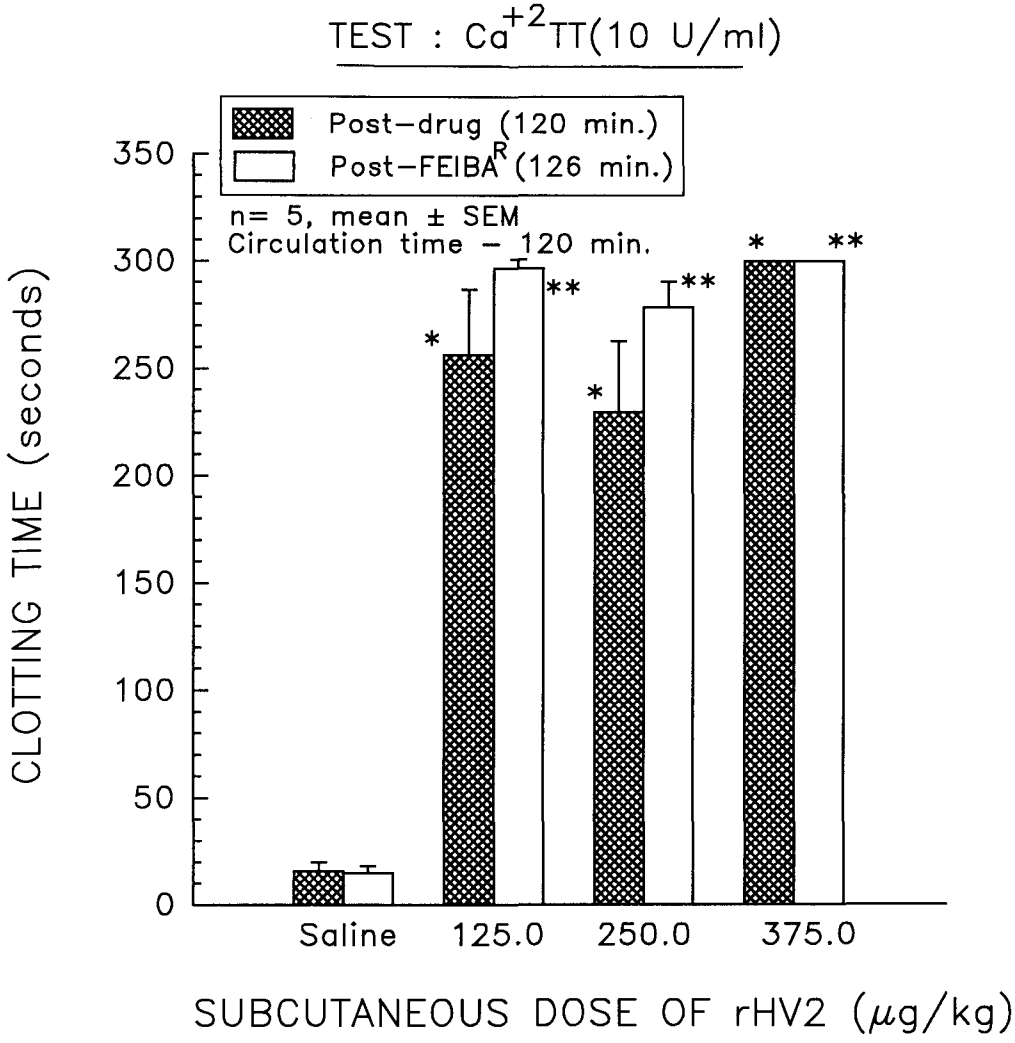


Figure 28. *Ex vivo* antithrombin activity of rHV2 using $Ca^{+2}TT$ (10 U/mL), after s.c. administration in rabbits. rHV2 was used in doses of 0, 125, 250 and 375 μg/kg in male, New Zealand White rabbits (n = 5). All data represent a mean of 5 responses each determined in duplicate ± SEM.
*, **- significant difference ($p < 0.05$) in $Ca^{+2}TT$ (10 U/mL) values compared to saline in post-drug and post-FEIBA® samples, respectively (one-way ANOVA followed by Tukey test).

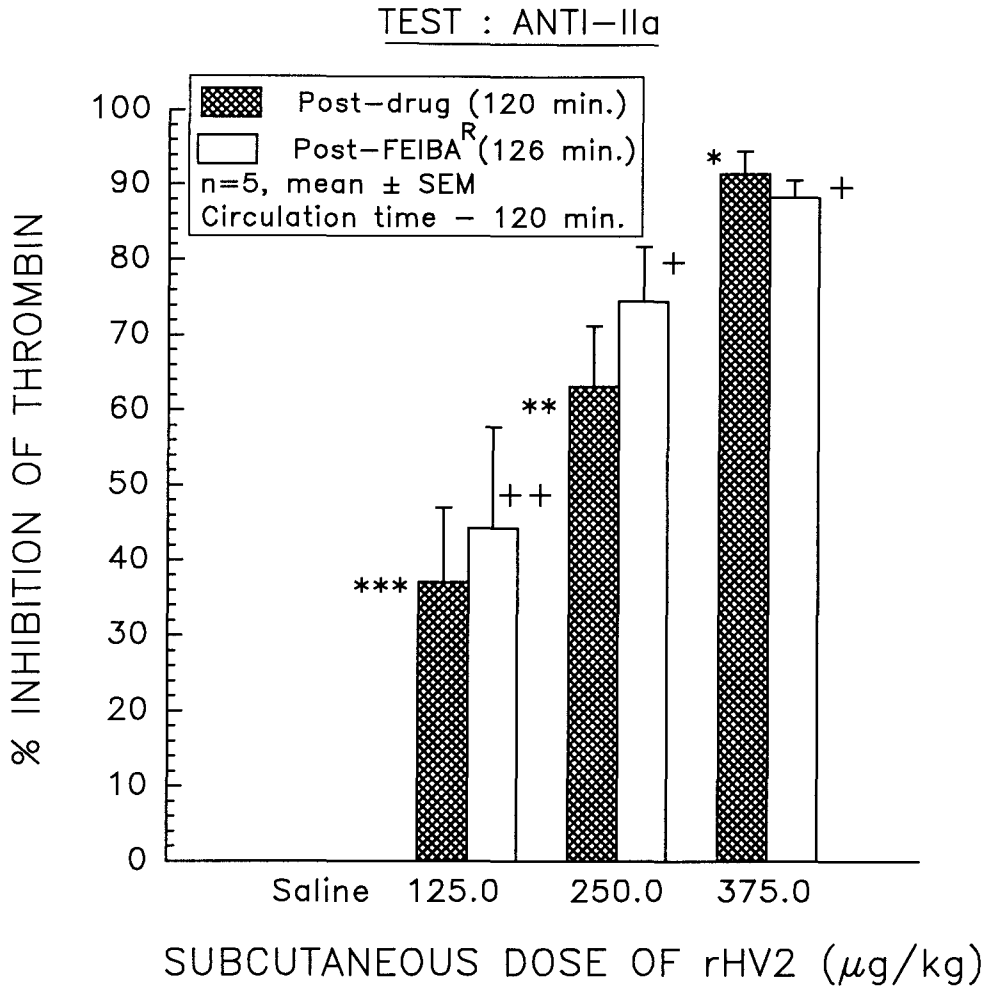


Figure 29. *Ex vivo* antithrombin activity of rHV2 using chromogenic anti-IIa assay, after s.c. administration in rabbits. rHV2 was used in doses of 0, 125, 250 and 375 μg/kg in male, New Zealand White rabbits (n=5). All data represent a mean of 5 responses each determined in duplicate ± SEM.

* - significant difference ($p<0.05$) in thrombin inhibitory activity compared to saline, 125 and 250 μg/kg rHV2 treated groups, ** - significant difference ($p<0.05$) from saline and 125 μg/kg rHV2 groups and *** - significant difference ($p<0.05$) from saline in post-drug samples; + - significant difference ($p<0.05$) from saline and 125 μg/kg rHV2 treated groups and + + - significant difference ($p<0.05$) from saline treated group in post-FEIBA® samples (one-way ANOVA followed by Tukey test).

the three doses in post-drug and post-FEIBA® samples (one-way ANOVA followed by Tukey test). Progressively increasing, dose dependent antithrombin activity was observed at 125, 250 and 375 μg/kg of rHV2. A thrombin inhibitory activity of approximately 92 ± 4% was seen after s.c. administration of 375 μg/kg of rHV2 (Figure 29).

Selection of appropriate s.c. dose for time course study. From the above results on *in vivo* clot scores and *ex vivo* coagulation testing and anti-IIa assay, a dose of 375 μg/kg was chosen for time course studies in rabbits after s.c. administration of rHV2 using the stasis thrombosis model. The results from these studies are described in the following sections.

2. Assessment of Hemorrhagic Activity of rHV2 using a Modified Rabbit Ear Blood Loss Model

The hemorrhagic activity of rHV2 was studied in a modified rabbit ear blood loss model as described in the "Materials, Methods and Experimental Protocols" chapter.

I.V. administration. A dose dependent increase in hemorrhagic activity was observed after i.v. administration of rHV2 in male, New Zealand White rabbits, as shown in Figure 30. A more linear increase in RBC loss was found with increasing doses after 5 minutes ($r = 0.97$) when compared to that after 15 minutes ($r = 0.80$). An apparent maximal bleeding response was obtained after administration of 1 mg/kg of rHV2 at both 5 and 15 minutes. No

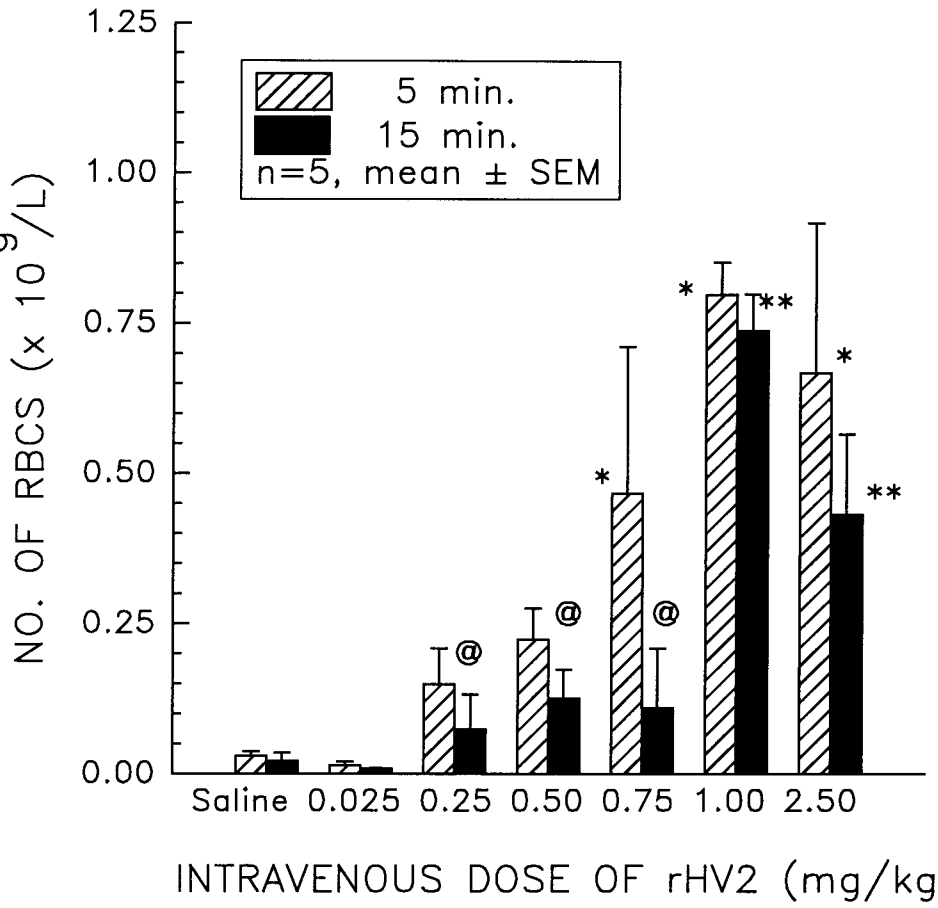


Figure 30. Hemorrhagic effects of rHV2 in a rabbit modified ear blood loss model after i.v. administration. Male, New Zealand White rabbits (n = 5) were administered with increasing i.v. doses of 0, 0.025, 0.25, 0.5, 0.75 1.0 and 2.5 mg/kg and blood loss was measured at 5 and 15 minutes. All data represent a mean of 5 responses each determined in duplicate ± SEM.

* - significant difference ($p < 0.05$) in RBC loss compared to saline, 0.025, 0.25 and 0.5 mg/kg rHV2 treated groups at 5 minutes, ** - significant difference ($p < 0.05$) from saline, 0.025, 0.25, 0.5 and 0.75 mg/kg rHV2 treated groups at 15 minutes (one-way ANOVA followed by Tukey test). @ - significant difference ($p < 0.05$) between RBC loss at 5 and 15 minutes at specific doses of rHV2 (paired t-test).

significant variations were found in blood loss with the administration of rHV2 doses of up to 0.5 mg/kg, i.v., when compared with saline treated group, after both circulation times. It is noteworthy that there was no difference in blood loss between saline treatment and antithrombotic i.v. dose (0.025 mg/kg) of rHV2, as determined from the studies using the modified stasis thrombosis model, after 5 and 15 minutes.

Significant differences ($p<0.05$) were found in blood loss after administration of much higher doses of rHV2 (0.75, 1 and 2.5 mg/kg, i.v.) when compared to the responses after saline and lower rHV2 doses, using a one-way ANOVA followed by multiple comparison using Tukey test, as illustrated in Figure 30. In the linear portion of the dose response curve, significant decreases ($p<0.05$) were found in blood loss at 15 minutes when compared to that at 5 minutes (0.25, 0.5 and 0.75 mg/kg) using paired t-test.

S.C. administration. Figure 31 depicts a similar dose dependent hemorrhagic activity after administration of increasing s.c. doses of rHV2. Linear increases in bleeding responses were obtained with increasing rHV2 doses at time periods of 1 hour ($r=0.98$) and 3 hours ($r=0.90$) after administration. As seen in the i.v. studies, there was no variation in the bleeding response between saline treatment and antithrombotic s.c. dose (0.375 mg/kg) of rHV2, as determined from the experiments using stasis thrombosis model, at both 1 and 3 hours. Significant increases in RBC loss

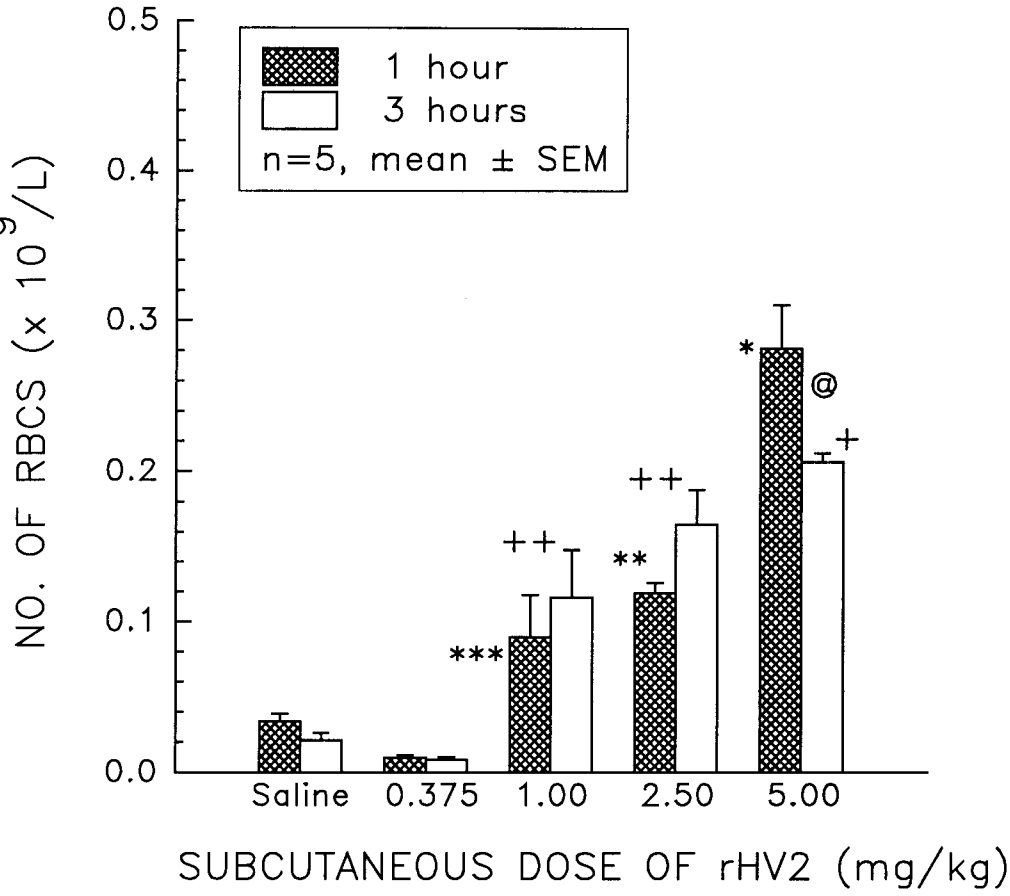


Figure 31. Hemorrhagic effects of rHV2 in a rabbit modified ear blood loss model after s.c. administration. Male, New Zealand White rabbits (n = 5) were administered with increasing s.c. doses of 0, 0.375, 1, 2.5 and 5 mg/kg and blood loss was measured at 1 and 3 hours. All data represent a mean of 5 responses each determined in duplicate ± SEM.

* - significant difference ($p<0.05$) in RBC loss compared to saline, 0.375, 1 and 2.5 mg/kg rHV2 treated groups at 1 hr, ** - significant difference ($p<0.05$) from saline and 0.375 rHV2 treated groups at 1 hr, *** - significant difference ($p<0.05$) from 0.375 mg/kg treated group at 1 hr, + - significant difference ($p<0.05$) from 0, 0.375 and 1 mg/kg rHV2 at 3 hrs and + + - significant difference ($p<0.05$) from 0 and 0.375 mg/kg doses at 3 hrs (one-way ANOVA followed by Tukey test). @ - significant difference ($p<0.05$) between RBC loss at 5 and 15 minutes at specific doses of rHV2 (paired t-test).

were seen after administration of 1 to 5 mg/kg doses of rHV2, when compared with corresponding saline- and 0.375 mg/kg dose of rHV2 treated groups, at 1 and 3 hours using a one-way ANOVA followed by Tukey test (Figure 31).

The bleeding response at 3 hours was approximately equivalent to, or higher than that observed at 1 hour after administration of all s.c. doses except 5 mg/kg after which there was a significant decrease in RBC loss at 3 hours (paired t-test).

It is important to emphasize that there was no significant variation in blood loss after administration with experimentally determined antithrombotic i.v. (0.025 mg/kg) and s.c. (0.375 mg/kg) doses of rHV2 when compared with blood loss from saline treated control rabbits using this rabbit model for ear blood loss.

3. Evaluation of Pharmacokinetics and Time Course of Antithrombotic Activity after rHV2 Administration in Rabbits

After the determination of a suitable antithrombotic i.v. (25 μg/kg) and s.c. (375 μg/kg) dose of rHV2, a time course study of antithrombotic activity was performed in rabbits. The results from these experiments are described below.

I.V. Administration. The time course of *in vivo* antithrombotic activity of rHV2 after i.v. administration is depicted in Figure 32. At the 10 minute

stasis interval, rHV2 exhibited a total, sustained antithrombotic activity up to almost 30 minutes, as indicated by clot scores of 0 at 5 and 15 minutes, and approximately 0.2 at 30 minutes. After a period of 30 minutes, the antithrombotic activity decreased gradually until clot scores of +3 and +4 were reached at 2 and 3 hours, respectively. However, at the 20 minute stasis time, the antithrombotic activity decreased markedly after the first 5 minutes. At this stasis time of 20 minutes, clot scores dropped from +1 at 5 minutes to +3.6 at 15 minutes, after which a constant response of almost equivalent to +4 was obtained, until 3 hours after administration of rHV2 (Figure 32).

In addition to *in vivo* antithrombotic responses, time course of *ex vivo* coagulation tests and amidolytic anti-IIa assay was also performed. The variations in APTT (OT) and APTT (Dade) values, with respect to time, are shown in Figure 33. APTT (OT) exhibited a mean peak increase from corresponding baseline values of a 118.67% at 3 minutes followed by a rapid decline to 34.07% at 5 minutes. After 5 minutes, APTT (OT) values fluctuated between 20% and 60% increases from baseline values until about 1.7 hours, after which there was no change from baseline values. APTT (Dade), on the other hand, showed a mean peak increase in clotting times of only 31.4% from baseline at 5 minutes, also shown in Figure 33. APTT

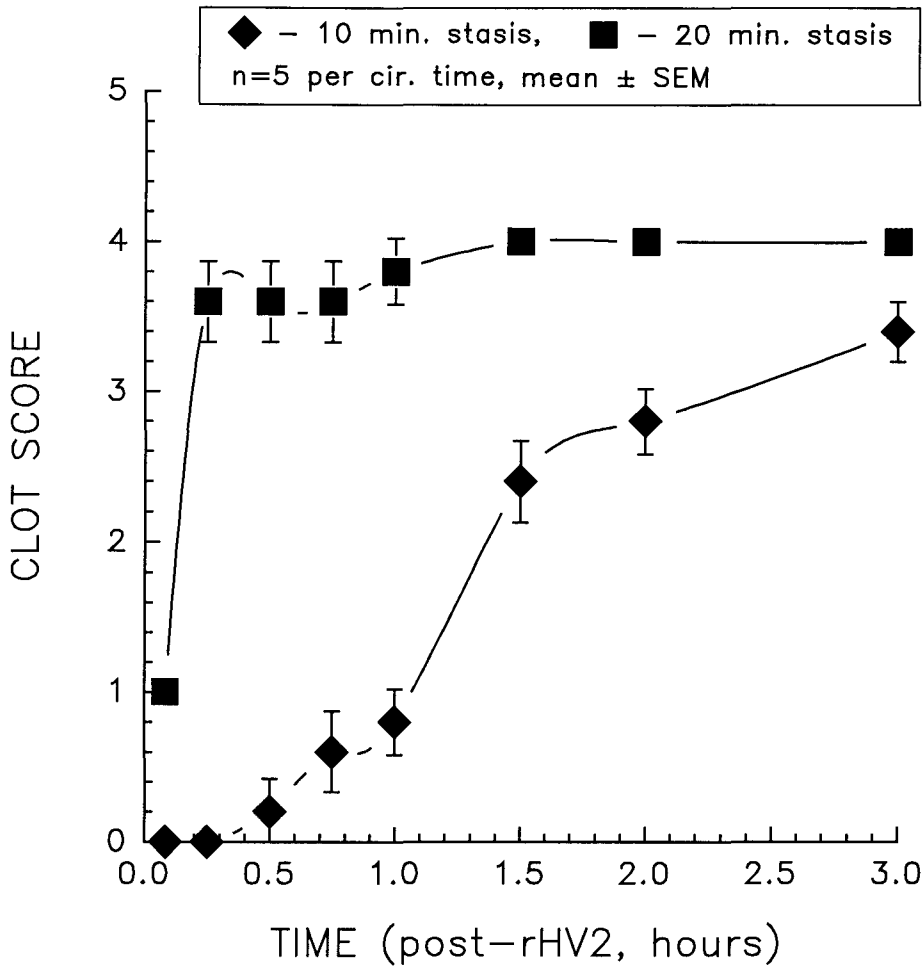


Figure 32. Time course of *in vivo* antithrombotic activity of rHV2 after i.v. administration (25 μg/kg). Male, New Zealand White rabbits (n = 5) were used in the modified jugular vein stasis thrombosis model. n = 5 rabbits were used for each circulation time. Clot scores were graded after a stasis time of 10 (◆) and 20 (■) minutes in the left and right jugular vein segments, respectively. All data represent the mean of 5 determinations ± SEM.

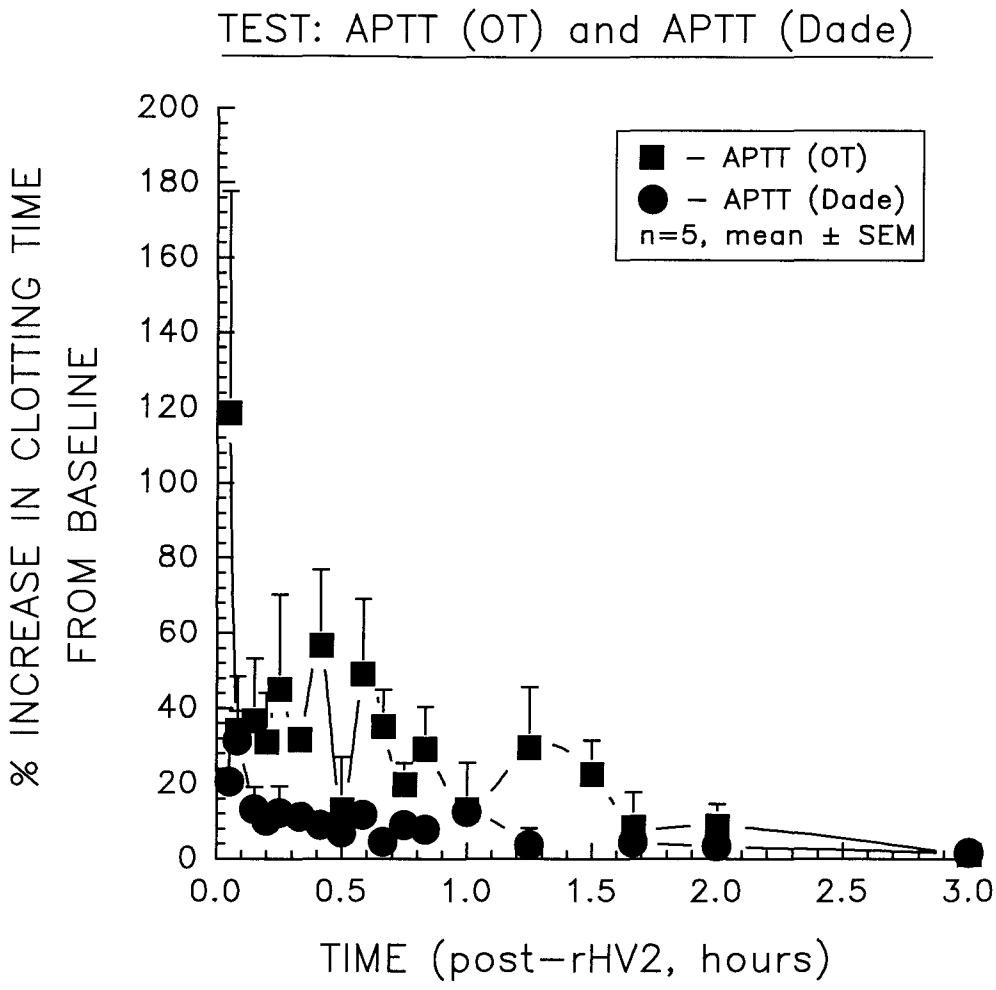


Figure 33. Time course of *ex vivo* antithrombin activity using coagulation tests, APTT (OT) { ■ } and APTT (Dade) { ● }. Male, New Zealand White rabbits (n = 5) were administered with an i.v. dose of 25 μg/kg of rHV2. All data represent the mean of 5 responses ± SEM, each determined in duplicate.

(Dade) values varied between 0 and 20% increases from baseline for the rest of the time period up to 3 hours.

The time course of $Ca^{+2}TT$ (10 U/mL) after i.v. administration is depicted in Figure 34. The decline in clotting times was rapid with a peak at 3 minutes (9-fold increase from baseline). Almost no anticoagulant effect of rHV2 was observed at about 1 hour, using this assay method.

Figure 35 depicts the time course of *ex vivo* thrombin inhibitory activity (chromogenic anti-IIa assay) after i.v. administration of rHV2. Peak thrombin inhibitory activity of about 85% was obtained 3 minutes after rHV2 administration followed by an anti-IIa activity of 75% at 5 minutes. Thereafter, a rapid decline in thrombin inhibitory activity was observed until only 10% of activity remained at 30 minutes, with almost no anti-IIa activity remaining after 1 hour (Figure 35).

The pharmacokinetics of rHV2 after administration of an i.v. dose of 25 μg/kg is illustrated in Figure 36. A maximum plasma rHV2 concentration (C_{max}) of 0.75 μg/mL was achieved at 3 minutes (t_{max}), as measured by the competitive ELISA method. The rHV2 concentrations decreased rapidly after that, until a level of $\approx$ 0.2 μg/mL was reached at 12 minutes. Thereafter, rHV2 concentrations remained steady between 0 and 0.2 μg/mL up to 3 hours (Figure 36).

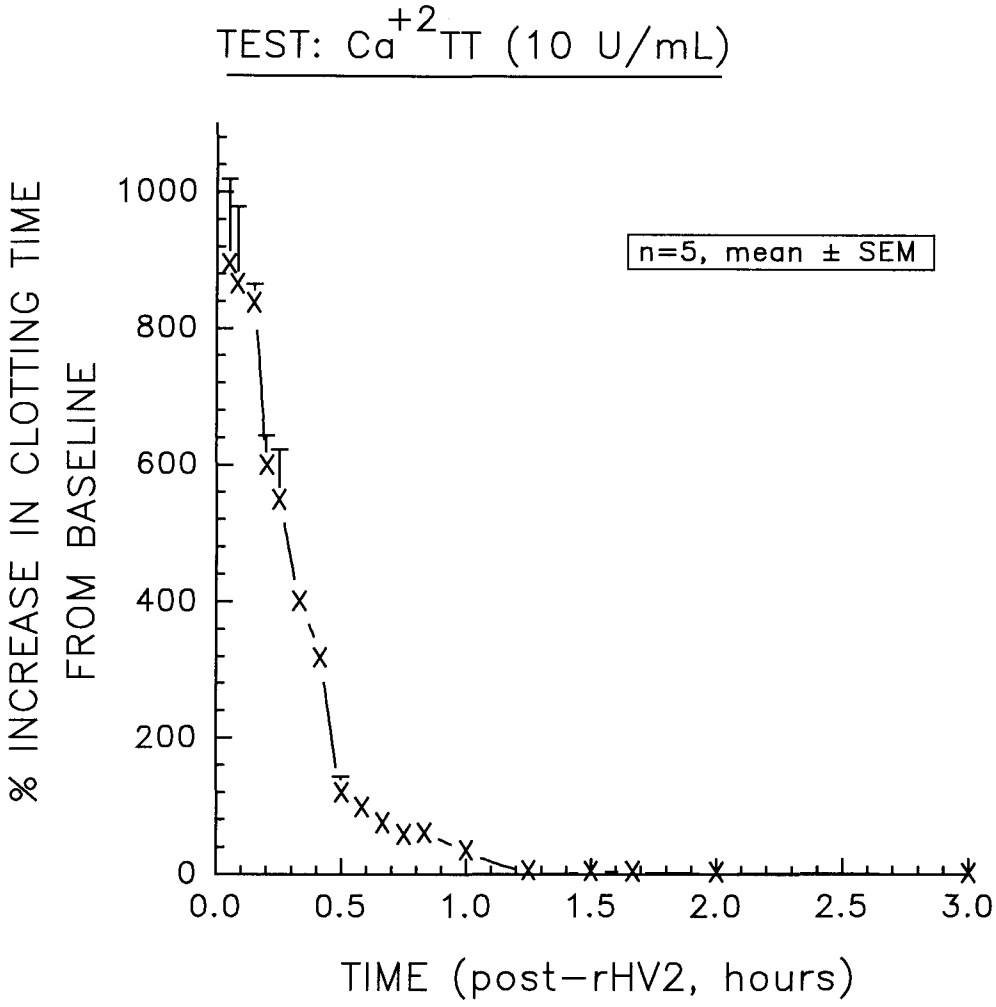


Figure 34. Time course of *ex vivo* antithrombin activity using $Ca^{+2}TT$ (10 U/mL) assay, after i.v. administration of rHV2 (25 μg/kg). n = 5 male, New Zealand White rabbits were used and blood samples drawn at regular intervals. All data represent mean of five responses each determined in duplicate ± SEM.

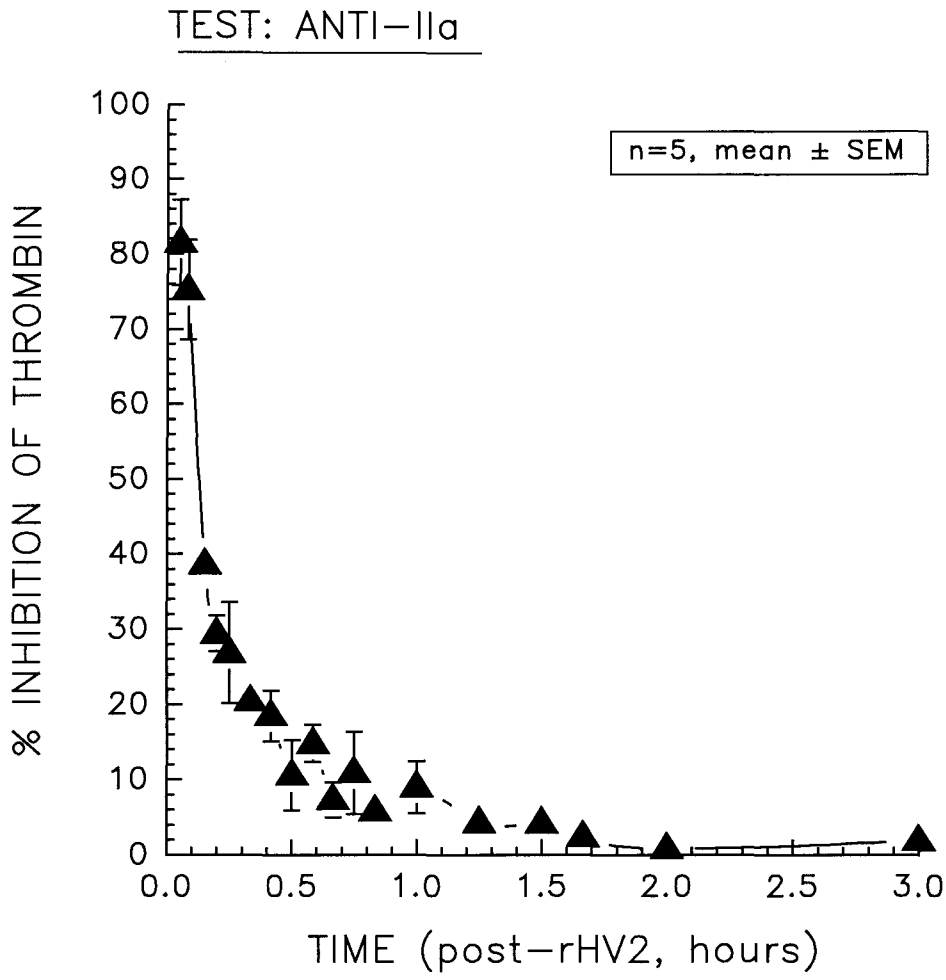


Figure 35. Time course of *ex vivo* antithrombin activity using the chromogenic anti-IIa assay, after i.v. administration of rHV2 (25 μg/kg). n = 5 male, New Zealand White rabbits were used and blood samples drawn at regular intervals. All data represent mean of five responses each determined in duplicate ± SEM.

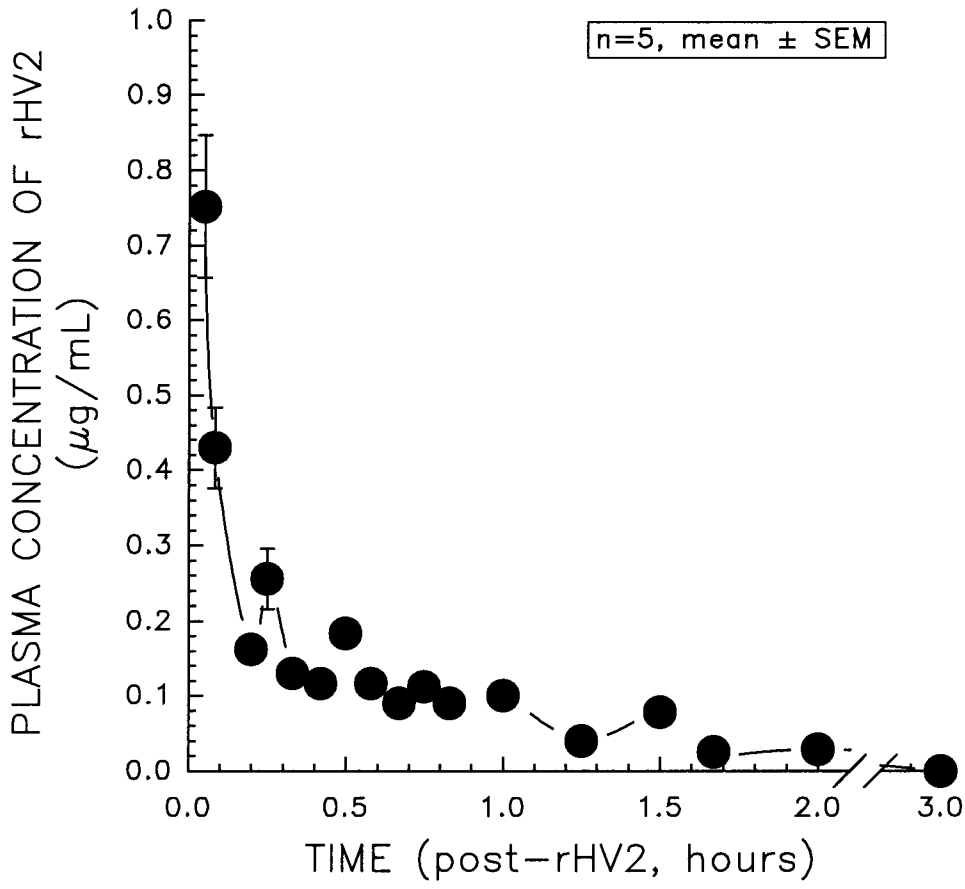


Figure 36. Pharmacokinetics of rHV2 after i.v. administration (25 μg/kg) in male, New Zealand White rabbits. Plasma concentrations were determined using competitive ELISA method. All data represent mean concentration from 5 rabbits, each determined in duplicate ± SEM.

Table 7 lists the pharmacokinetic parameters obtained by non-compartmental analysis after single dose i.v. administration and s.c. administration of rHV2 in rabbits. In the terminal phase after i.v. administration, the elimination rate constant (k_e) and the terminal elimination half-life ($t_{1/2}$) were 0.76 hr^{-1} and 0.91 hr (55 minutes), respectively. The area under the plasma concentration-time curve up to 3 hours (AUC_{0-3}) was found to be 0.24 μg-hr/mL. This value for the $AUC_{0-\infty}$ did not change when extrapolated to infinity. The area under the first moment curve up to 3 hours ($AUMC_{0-3}$) was 0.16 μg-hr^2/mL, which also remained unchanged when extrapolated to ∞.

The mean residence time of rHV2 (MRT) after i.v. administration was 0.66 hr (40 minutes), as indicated in Table 7. The total clearance rate (Cl_{tot}) of rHV2 in rabbits was found to be 104 mL/hr/kg. The volume of distribution (V_d) was 137 mL/kg (Table 7).

TABLE 7

PHARMACOKINETIC PARAMETERS OBTAINED AFTER SINGLE DOSE I.V. (25 μg/kg) AND S.C. (375 μg/kg) ADMINISTRATION OF rHV2 IN RABBITS

Parameter	I.V.Dosing	S.C.Dosing
Cmax (μg/mL)	0.75	0.38
t_{max} (min)	3.00	220.00
k_e (hr^{-1})	0.76	1.78
$t_{1/2}$ (hr)	0.91	0.39
$AUC_{0-\infty}$ (μg-hr/mL)	0.24	1.61
$AUMC_{0-\infty}$ (μg-hr^2/mL)	0.16	5.49
MRT (hr)	0.66	3.41
Cl_{tot} (mL/hr/kg)	104.17	104.16
V_d (mL/kg)	137.07	58.52

Integration of pharmacokinetics with pharmacodynamics of rHV2 after i.v. administration. A correlation analysis was performed between plasma rHV2 concentrations determined by the ELISA method and *ex vivo* antithrombotic activity. Figure 37 describes this relationship in the form of a sigmoidal curve. Clot scores obtained at the 10 minute stasis interval were plotted against plasma rHV2 concentrations obtained at the corresponding circulation time of rHV2. At very low rHV2 concentrations, little or no antithrombotic activity was obtained (clot scores of +3 and 3.4 at 2 and 3 hours, respectively). As the rHV2 concentrations increased in plasma, stronger antithrombotic activity was obtained, until total prevention of thrombosis occurred at 0.25 μg/mL (Figure 37). In the linear portion of the curve, a significant negative correlation was obtained between available plasma concentrations and antithrombotic activity (Pearson correlation coefficient, $r = -0.93$).

Figure 38 depicts the relationship between plasma concentrations obtained after i.v. administration of rHV2 and corresponding *ex vivo* antithrombin activity determined from coagulation tests and chromogenic substrate assay. The most significant correlation between indirect pharmacodynamics and direct pharmacokinetics of rHV2 was obtained with the chromogenic substrate assay ($r = 0.94$). The results from APTT (OT) and APTT (Dade) coagulation tests correlated poorly with corresponding plasma concentrations with $r = 0.81$ and $r = 0.77$, respectively. A significant

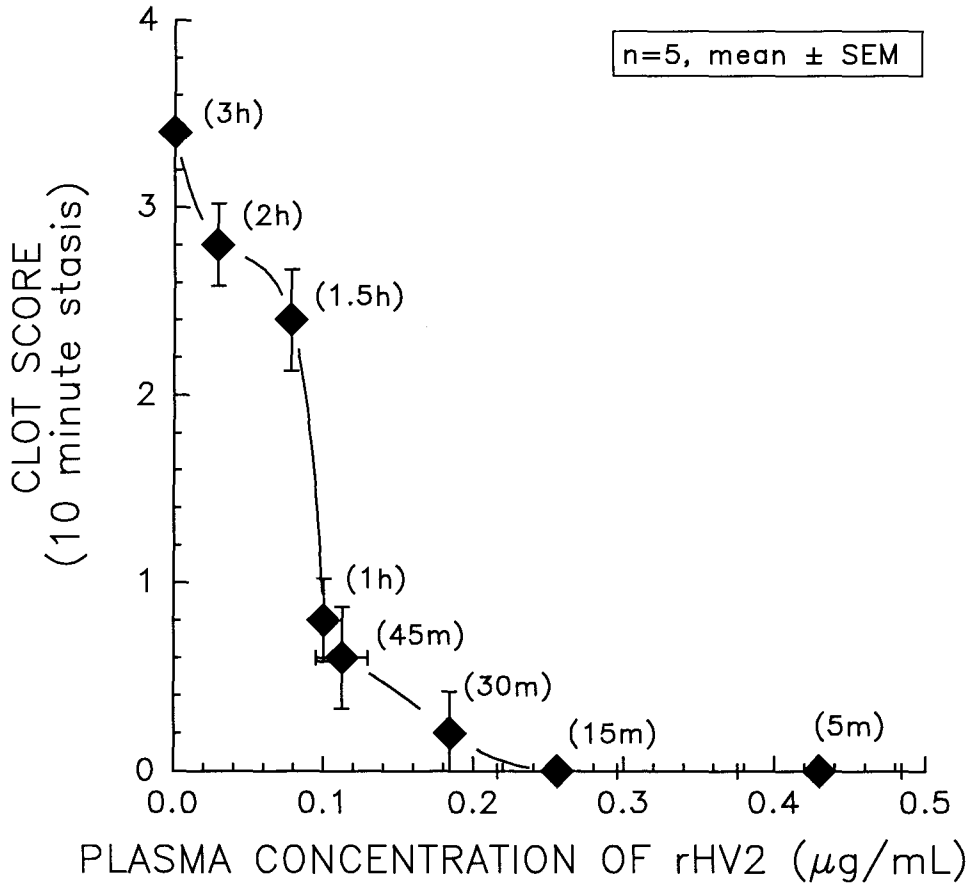


Figure 37. Correlation between plasma concentrations of rHV2 and *in vivo* antithrombotic activity of rHV2 after i.v. administration in rabbits. rHV2 was administered in a dose of 25 μg/kg in male, New Zealand White rabbits and clot scores were graded. Numbers in parentheses indicate circulation time of rHV2 in hours (h) or minutes (m). All data represent a mean of 5 determinations ± SEM.

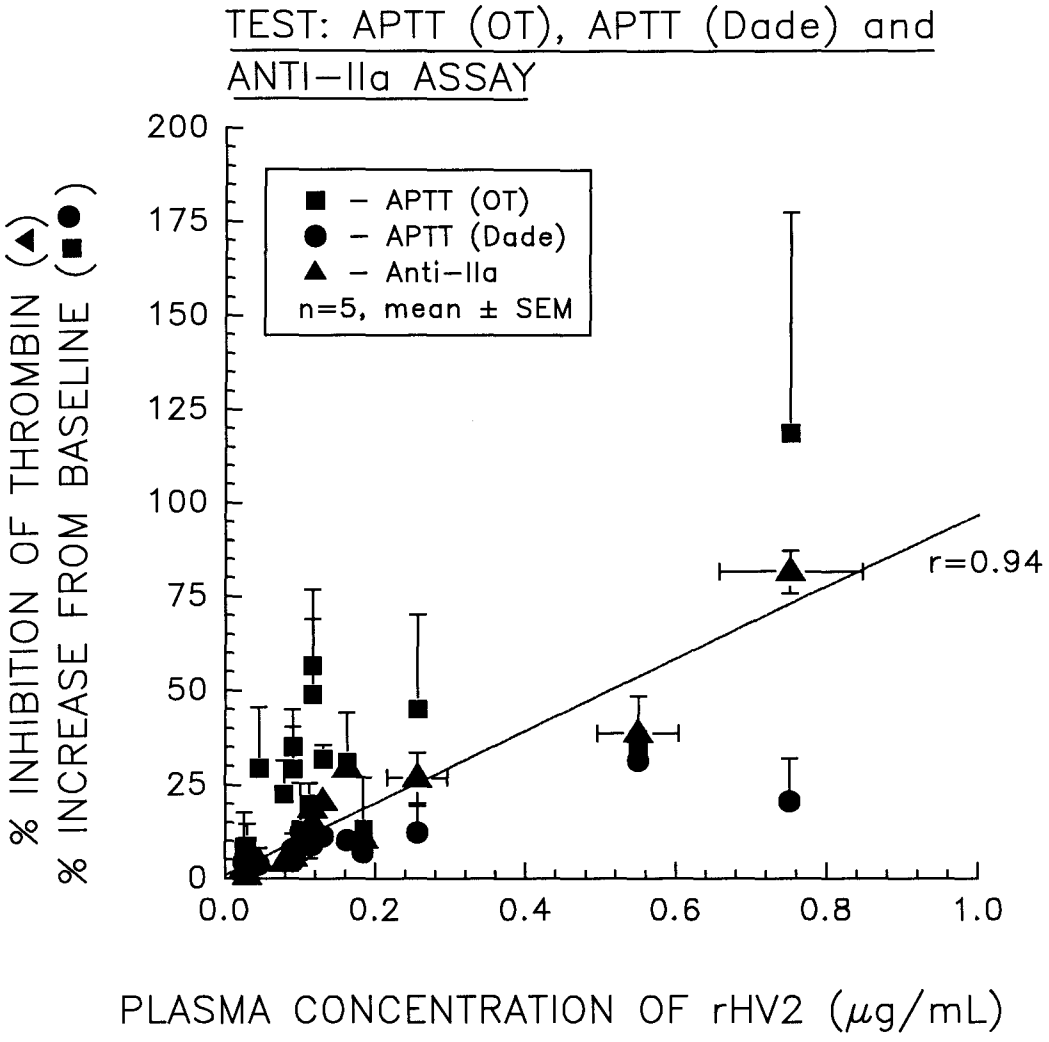


Figure 38. Relationship between plasma concentrations of rHV2 and *ex vivo* antithrombin activity, after i.v. administration in rabbits. Male, New Zealand White rabbits (n = 5) were administered with an i.v. dose 25 μg/kg of rHV2. The tests performed include APTT (OT) { ■ }, APTT (Dade) { ● } and chromogenic substrate assay { ▲ }. All data represent the mean of 5 determinations ± SEM, each determined in duplicate.

correlation (r = 0.87) was obtained between $Ca^{+2}TT$ (10 U/mL) and corresponding plasma rHV2 concentrations (Figure 39).

S.C. Administration. rHV2 was used at a dose of 375 μg/kg to determine the time course of antithrombotic activity after s.c. administration. The changes in *in vivo* antithrombotic activity with respect to time after s.c. administration of rHV2 are illustrated in Figure 40. At the 10 minute stasis time, a complete inhibition of clot formation was obtained almost throughout the time period up to 6 hours, as illustrated by clot scores of 0 to +0.5. A mean clot score of about +2.5 was obtained at 9 hours. On the other hand, when stasis was performed for 20 minutes, a "U" shaped curve was obtained exhibiting peak antithrombotic activity at 2 hours (clot score of almost 0). There was a steady progressive increase in antithrombotic activity of rHV2 up to 2 hours, after which the activity dropped markedly to clot score of +2.8 at 2.5 hours. Thereafter, the clot scores gradually increased to approximately +3.5 (Figure 40).

Figure 41 illustrates the time course of results from *ex vivo* coagulation tests, APTT (OT) and APTT (Dade). A 50% increase in clotting time was seen using APTT (Dade) about 30 minutes after drug administration, after which the responses fluctuated between 0 and 40% increases from baseline without exhibiting any time dependency. On the other hand, APTT (OT) showed a distinct peak in clotting time at 4 hours after rHV2 administration. Initially,

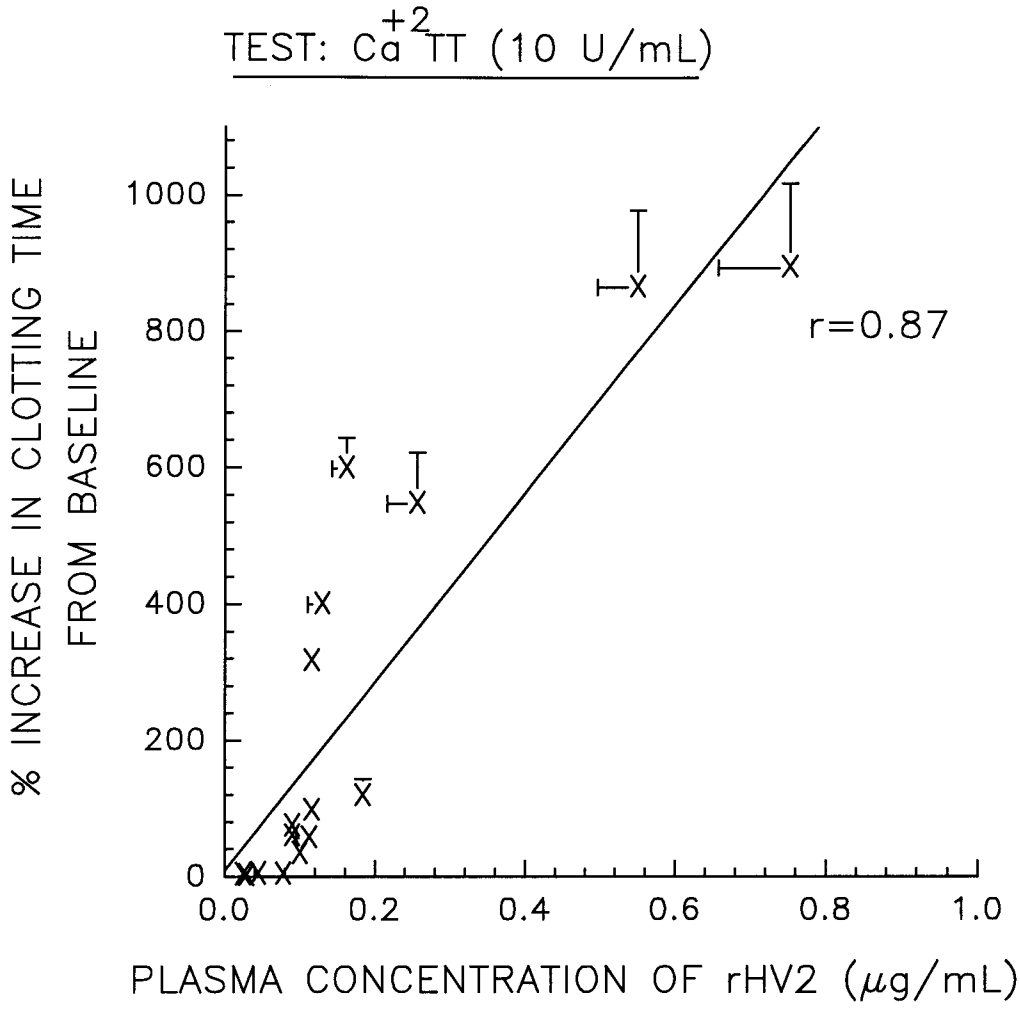


Figure 39. Correlation between plasma concentrations of rHV2 and *ex vivo* antithrombin activity using $Ca^{+2}TT$ (10 U/mL), after i.v. administration in rabbits. Male, New Zealand White rabbits (n = 5) were administered with an i.v. dose 25 μg/kg of rHV2. All data represent the mean of 5 determinations ± SEM, each determined in duplicate.

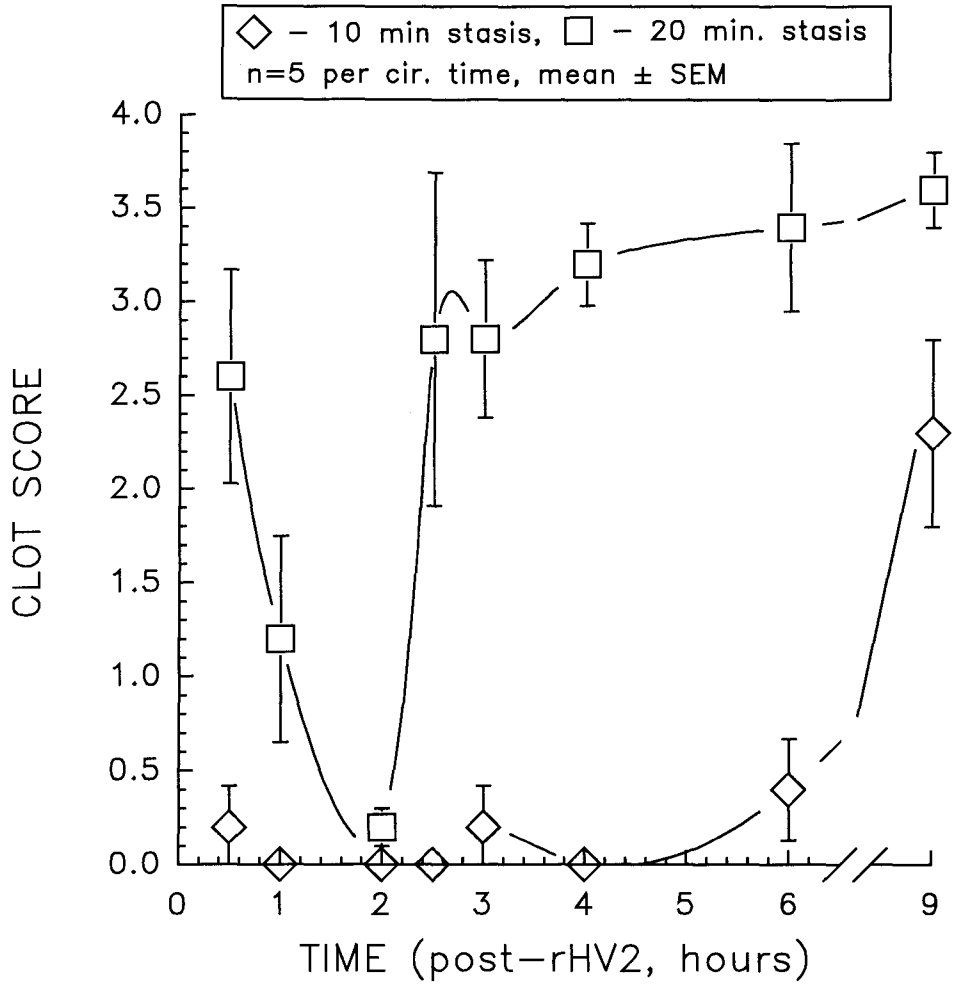


Figure 40. Time course of *in vivo* antithrombotic activity of rHV2 after s.c. administration in rabbits. rHV2 was administered in a s.c. dose of 375 μg/kg in male, New Zealand White rabbits (n = 5 per circulation time). Clot scores were graded after stasis for 10 and 20 minutes in the left and right jugular vein segments, respectively. All data are mean responses from 5 rabbits, each determined in duplicate ± SEM.

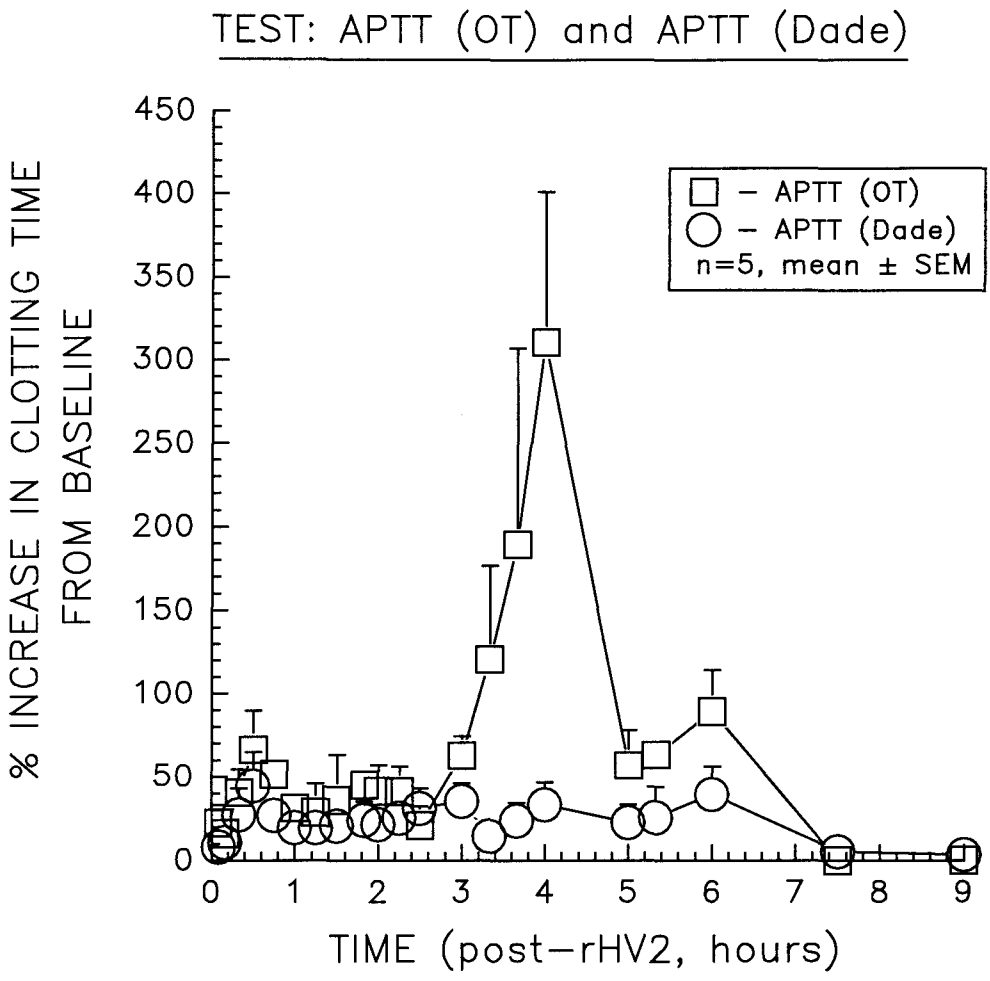


Figure 41. Time course of *ex vivo* antithrombin activity of rHV2 after s.c. administration in rabbits. Figure depicts results from coagulation tests: APTT (OT) { □ } and APTT (Dade) { ○ }. All data represent mean response from 5 rabbits, each determined in duplicate ± SEM.

APTT (OT) values fluctuated between 0 and 70% increases from corresponding baseline values up to 3 hours, after which the responses increased progressively until a peak was reached at 4 hours, as shown in Figure 41. After 4 hours, APTT (OT) values declined rapidly until steady state levels were reached.

The time course of *ex vivo* clotting time using $Ca^{+2}TT$ (10 U/mL) is depicted in Figure 42. Thirty minutes after rHV2 administration, $Ca^{+2}TT$ values increased progressively up to about a 21-fold increase from corresponding baseline values. During the period between 30 minutes and 4 hours, the $Ca^{+2}TT$ values fluctuated between 10- and 21-fold increases from baseline values. After 4 hours, the responses dropped steadily to baseline values.

The changes in *ex vivo* antithrombin activity measured using chromogenic substrate assay, with respect to time is shown in Figure 43. After s.c. administration of rHV2, the thrombin inhibitory activity of this agent increased steadily with increases in circulation time of rHV2 until an anti-IIa activity of about 82% was reached at 1 hour. The anti-IIa activity remained constant between 80 and 90% between the time period of 1 to 4 hours. Thereafter, the responses decreased rapidly until no anti-IIa activity remained at 7.5 hours (Figure 43).

The pharmacokinetics of rHV2 after s.c. administration of 375 μg/kg of rHV2 is described in Figure 44. Plasma concentrations of rHV2 were

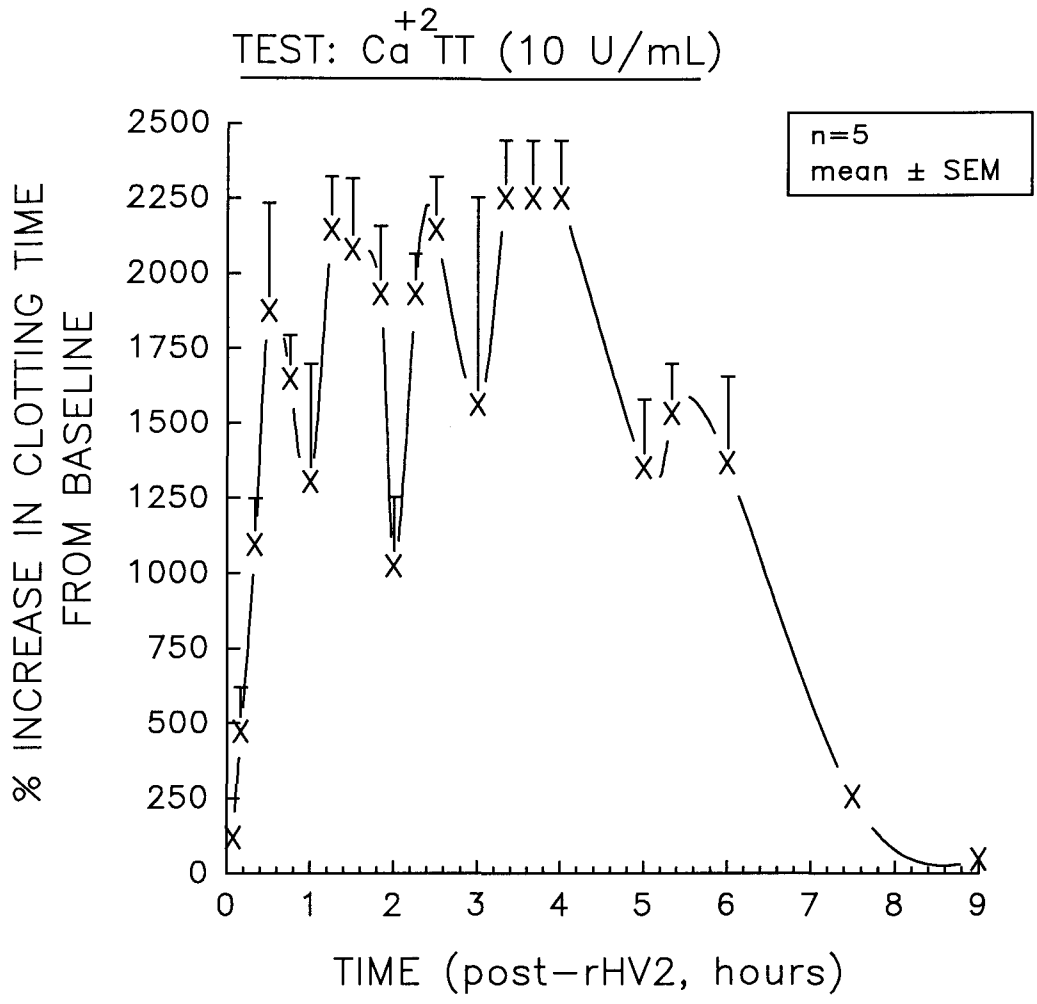


Figure 42. Time course of *ex vivo* antithrombin activity of rHV2 using Ca^{+2}TT (10 U/mL), after s.c. administration in rabbits. Male, New Zealand White rabbits (n=5) were subcutaneously administered with 375 μg/kg of rHV2. All data represent the mean response from 5 rabbits, each determined in duplicate, ± SEM.

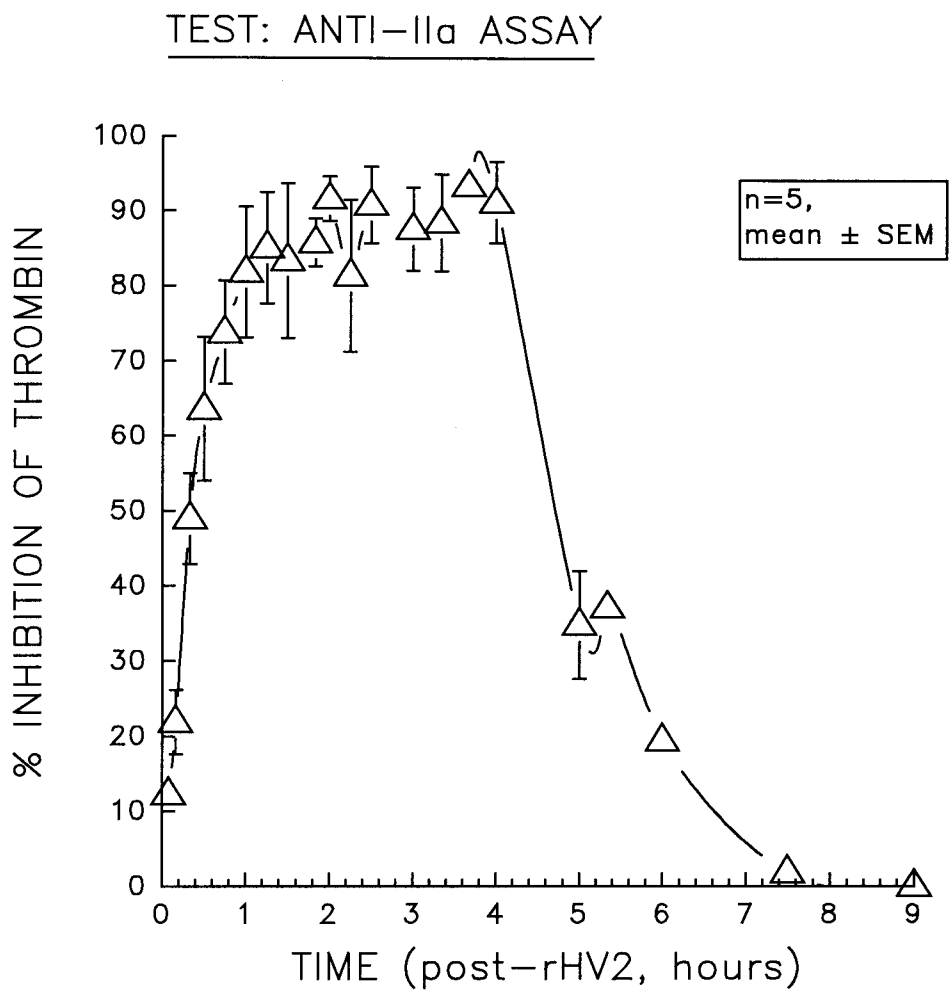


Figure 43. Time course of *ex vivo* antithrombin activity of rHV2 using chromogenic anti-IIa assay, after s.c. administration in rabbits. Male, New Zealand White rabbits (n = 5) were subcutaneously administered with 375 μg/kg of rHV2. All data represent the mean response from 5 rabbits, each determined in duplicate, ± SEM.

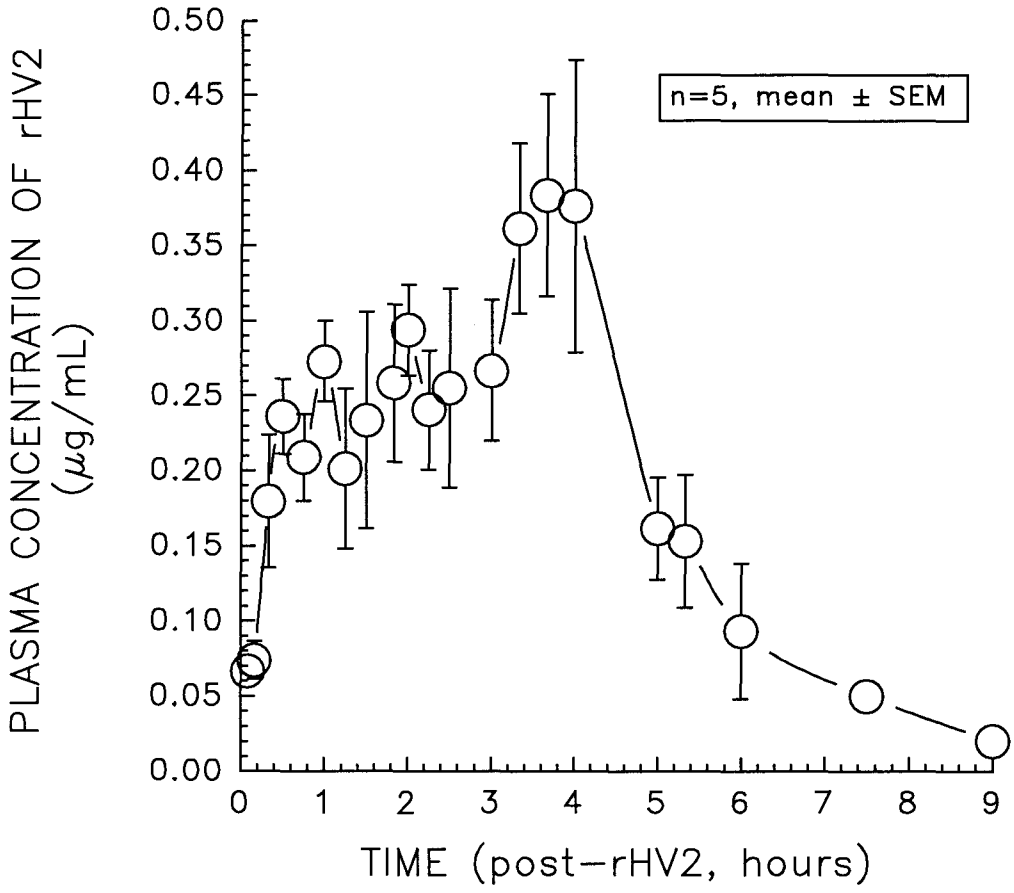


Figure 44. Pharmacokinetics of rHV2 after s.c. administration in rabbits. Male, New Zealand White rabbits (n = 5) were administered with 375 μg/kg of rHV2. Plasma rHV2 concentrations were determined using a competitive ELISA method. All data represent the mean of concentrations from five rabbits, each determined in duplicate ± SEM.

determined by the competitive ELISA method, as described previously. An absorption phase, a peak and an apparent terminal elimination phase were observed in the plasma rHV2 concentration-time curve. A peak rHV2 concentration (C_{max}) of $\approx$ 0.38 μg/mL was obtained at 220 minutes (t_{max}) after rHV2 administration. After the peak, rHV2 concentrations decreased steadily until a very low concentration ($\approx$ 0.05 μg/mL) was found at 7.5 hours.

The pharmacokinetic parameters obtained after s.c. administration of 375 μg/kg of rHV2 are listed in Table 7. The C_{max} obtained (0.38 μg-hr/mL) was only about half of that obtained after i.v. administration. Once the peak was reached, the elimination rate (k_e) of rHV2 was fast at 1.78 hr^{-1}. The terminal elimination half-life ($t_{1/2}$) was found to be 0.39 hr (23.40 minutes) which was less than half of that obtained after i.v. administration. The areas under the plasma concentration-time curve ($AUC_{0-\infty}$) and first moment curve ($AUMC_{0-\infty}$) were found to be 1.61 μg-hr/mL and 5.49 μg-hr^2/mL, respectively.

After s.c. administration, the mean residence time (MRT) of rHV2 was 3.4 hours which was about 5 times that obtained after i.v. administration, as shown in Table 7. The total clearance rate (Cl_{tot}) after s.c. administration was almost identical to that obtained after i.v. administration, i.e. 104 mL/hr/kg. The apparent volume of distribution (V_d) was about half of that obtained after i.v. administration i.e. 58 mL/kg. The relative bioavailability (F) after s.c.

administration was found be 44.72%, when compared to i.v. administration.

Integration of pharmacokinetics with pharmacodynamics of rHV2 after s.c. administration. A correlation analysis of plasma rHV2 concentrations determined from the ELISA method and results from *in vivo* and *ex vivo* assay methods was performed. After s.c. administration, the clot scores obtained using the jugular vein stasis thrombosis model were plotted against plasma rHV2 concentrations obtained at specific circulation times of rHV2. This relationship is illustrated in Figure 45, with arrow heads pointing toward the direction of increasing circulation times of rHV2. At 0.5 hr (and rHV2 concentration of 0.24 μg/mL) the clot score was +2.4 which decreased to +1.2 and +0.2 at 1 hour (rHV2 level of 0.27 μg/mL) and 2 hours (rHV2 level of 0.3 μg/mL) after rHV2 administration, respectively. Hence, peak antithrombotic activity was obtained at 2 hours at a plasma concentration of 0.3 μg/mL of rHV2, as shown in Figure 45. After 2 hours, the antithrombotic activity decreased markedly to a clot score of +2.8 at 2.5 and 3 hours (rHV2 level of 0.26 μg/mL). It was interesting to note that at 4 hours, a clot score of +3.2 was obtained even though the plasma concentration was at its apparent peak, i.e. 3.8 μg/mL. After 4 hours, the antithrombotic activity decreased progressively with clot scores of +3.4 and +3.6 at 6 and 9 hours, respectively.

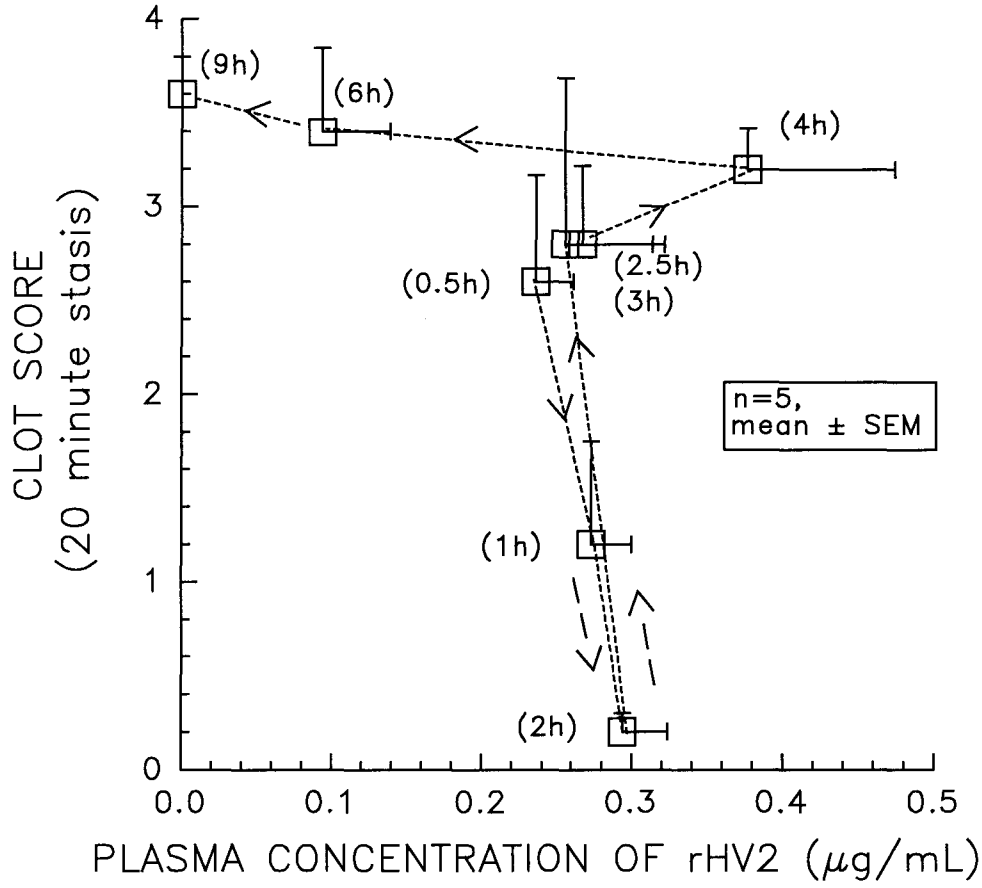


Figure 45. Relationship between plasma concentrations of rHV2 and *in vivo* anti-thrombotic activity using modified jugular vein stasis thrombosis model. Male, New Zealand White rabbits (n = 5) were subcutaneously administered with 375 μg/kg of rHV2. Numbers in parentheses indicate circulation time of rHV2 in hours (h). Arrow heads point toward direction of increasing circulation times of rHV2.

All data represent the mean response from 5 rabbits, each determined in duplicate, ± SEM.

The relationship between the results from coagulation tests, APTT(OT) and APTT (Dade) with plasma rHV2 concentrations is shown in Figure 46. Relatively poor correlations were obtained between APTT (OT) and APTT (Dade) values and corresponding plasma concentrations, as shown by correlation coefficient values of 0.60 and 0.19, respectively. On the other hand, the results from $Ca^{+2}TT$ (10 U/mL) correlated better with *ex vivo* plasma rHV2 concentrations ($r = 0.73$), though not as significant as that obtained after i.v. administration (Figure 47). Figure 48 illustrates the relationship between the results from the *ex vivo* chromogenic anti-IIa assay and those from the ELISA method. The most significant correlation after s.c. administration of rHV2 was obtained with this assay ($r = 0.89$).

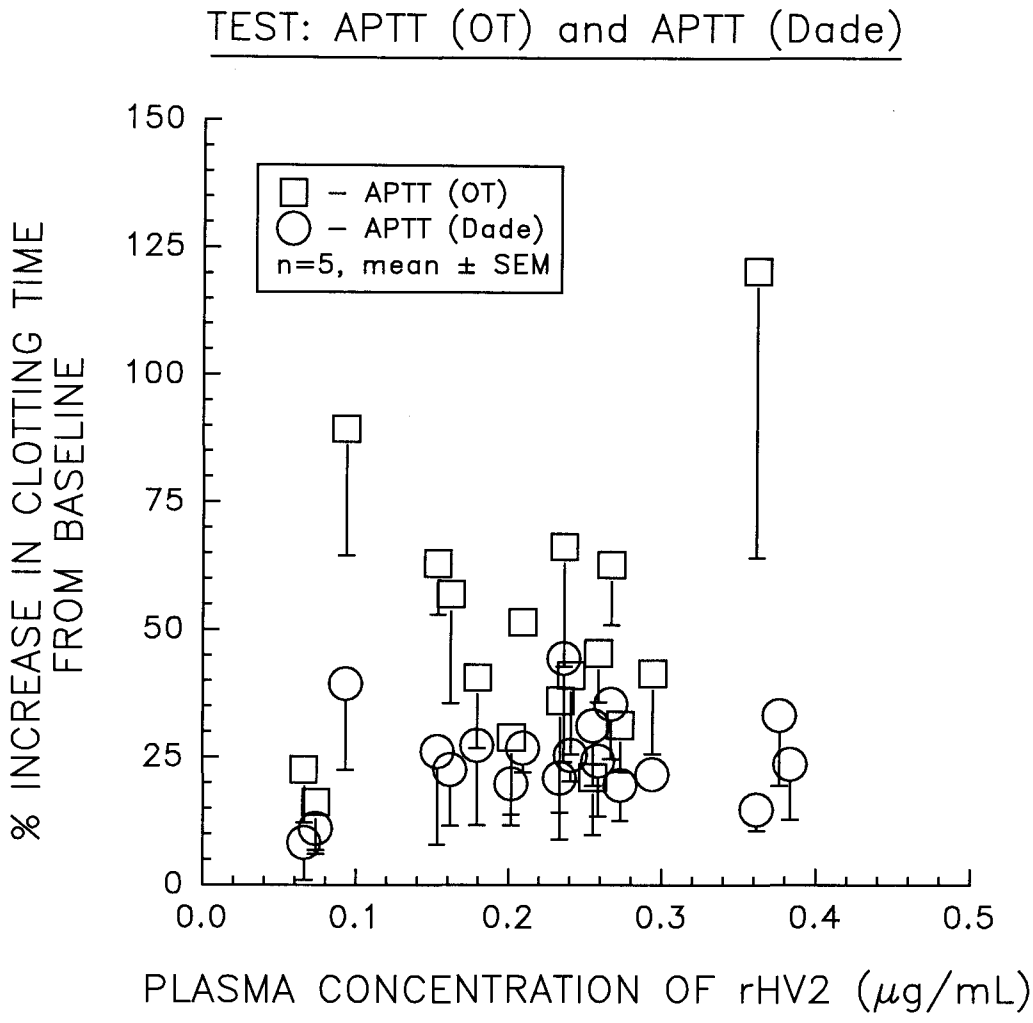


Figure 46. Relationship between plasma concentrations of rHV2 and coagulation tests, APTT (OT) { □ } and APTT (Dade) { ○ } after s.c. administration. Male, New Zealand White rabbits (n = 5) were administered with 375 μg/kg of rHV2. All data represent the mean response from 5 rabbits, each determined in duplicate, ± SEM.

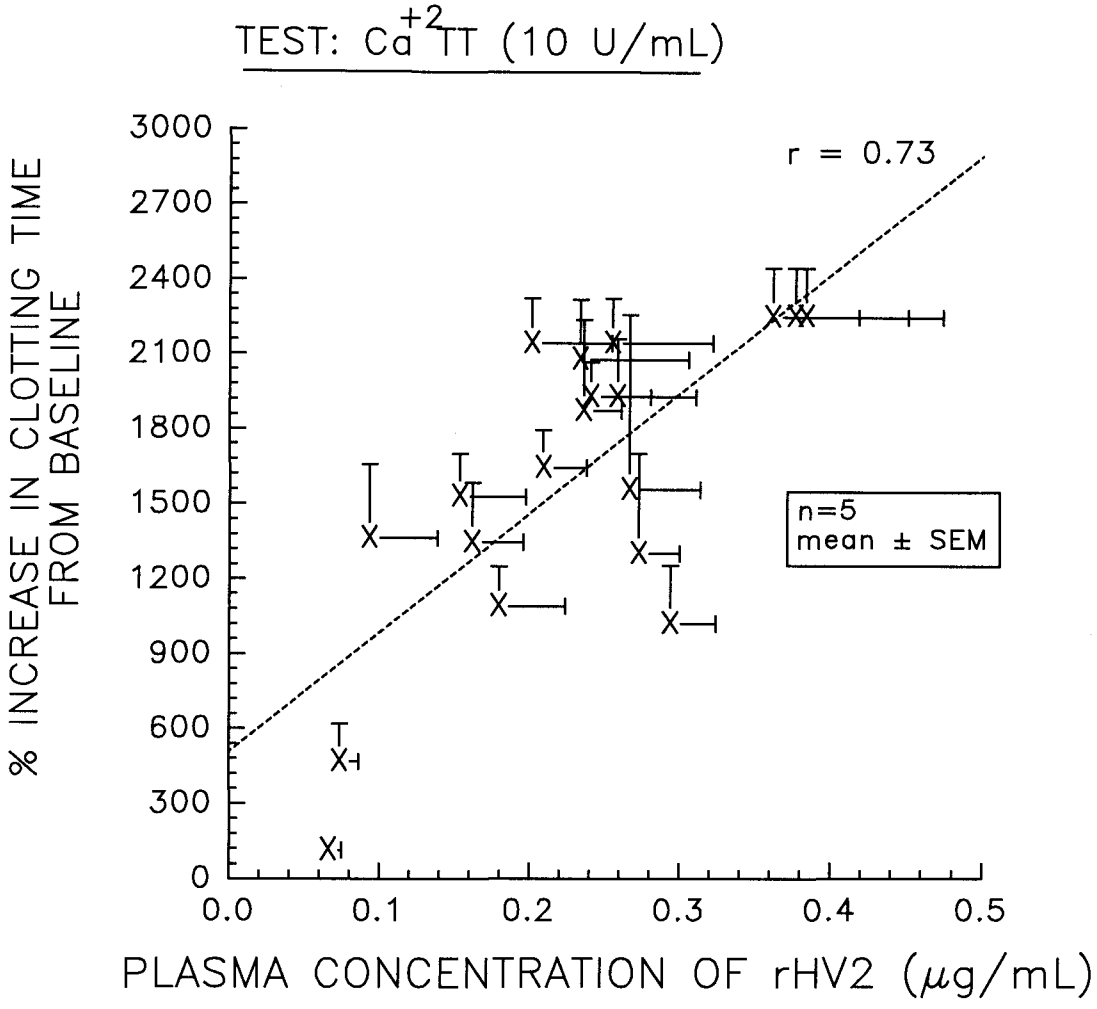


Figure 47. Correlation between plasma concentrations of rHV2 and coagulation test, $Ca^{+2}TT$ (10 U/mL) after s.c. administration. Male, New Zealand White rabbits (n = 5) were administered with 375 μg/kg of rHV2. All data represent the mean response from 5 rabbits, each determined in duplicate, ± SEM.

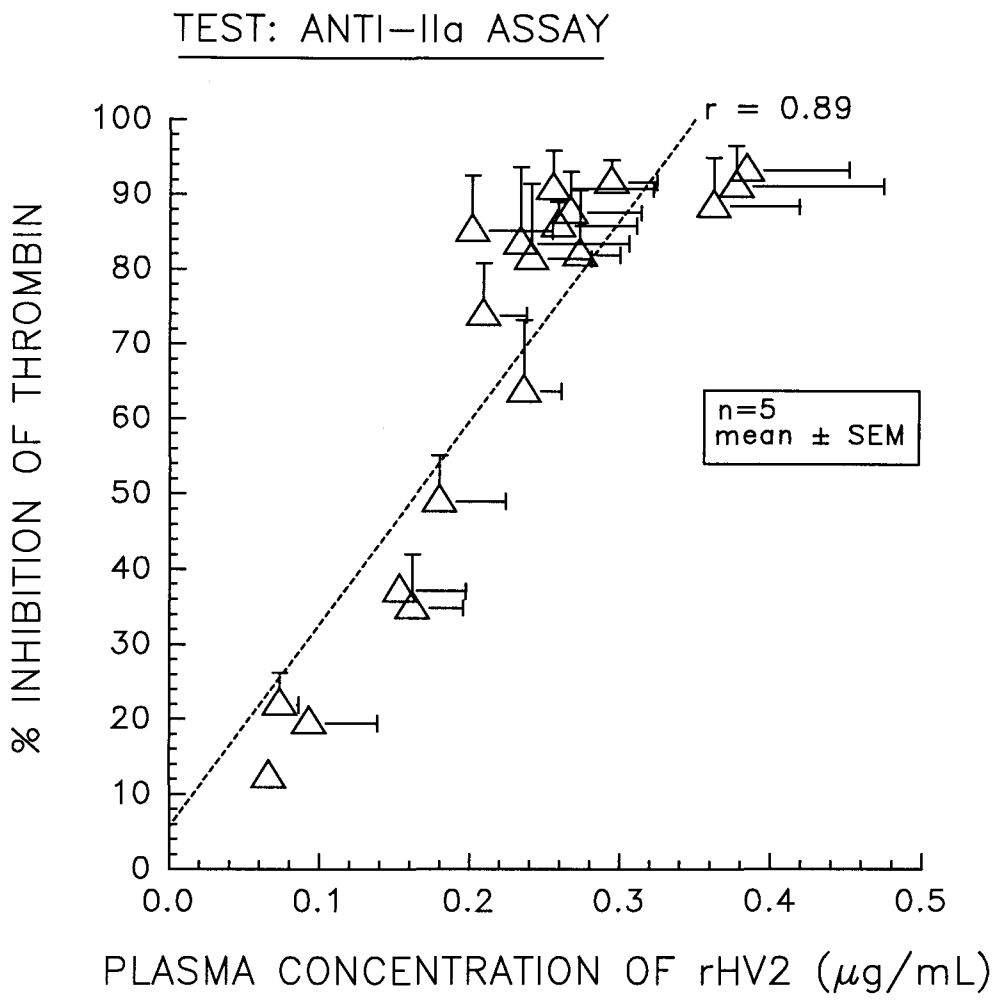


Figure 48. Correlation between plasma concentrations of rHV2 and *ex vivo* anti-thrombin activity using chromogenic anti-IIa assay, after s.c. administration of rHV2. Male, New Zealand White rabbits (n = 5) were subcutaneously administered with 375 μg/kg of rHV2. All data represent the mean response from 5 rabbits, each determined in duplicate, ± SEM.

4. Determination of Pharmacokinetic Characteristics and *Ex Vivo* Antithrombin Activity of rHV2 in Rats

The pharmacokinetics of rHV2 was studied in rats after administration of increasing i.v. and s.c. doses, as described in the chapter on "Materials, Methods and Experimental Protocols".

I.V. Administration. rHV2 was administered in i.v. doses of 0.1, 0.4 and 0.5 mg/kg in male, Sprague-Dawley rats (n = 8 per treatment group and per time schedule, composite data) via the tail vein. Plasma concentrations of rHV2 at specified time points were determined using the competitive ELISA method. Figure 49 depicts the composite plasma concentration-time curve after i.v. administration of rHV2 in rats. A dose dependent increase in plasma rHV2 concentrations was observed with increasing rHV2 doses. After administration of each dose, rHV2 distributed quickly, after which there was a more gradual decline in plasma concentrations reaching almost zero within the first 45 minutes.

Table 8 lists the pharmacokinetic parameters after administration of each dose, as determined by non-compartmental analysis. The time (t_{max}) to reach maximum plasma concentration (C_{max}) was 3 minutes after each dose. The C_{max} values were $\approx$ 187, 579 and 821 ng/mL after administration of 0.1, 0.4 and 0.5 mg/kg doses, respectively (Table 8, Figure 49). A dose dependent increase in area under the plasma concentration-time curve values

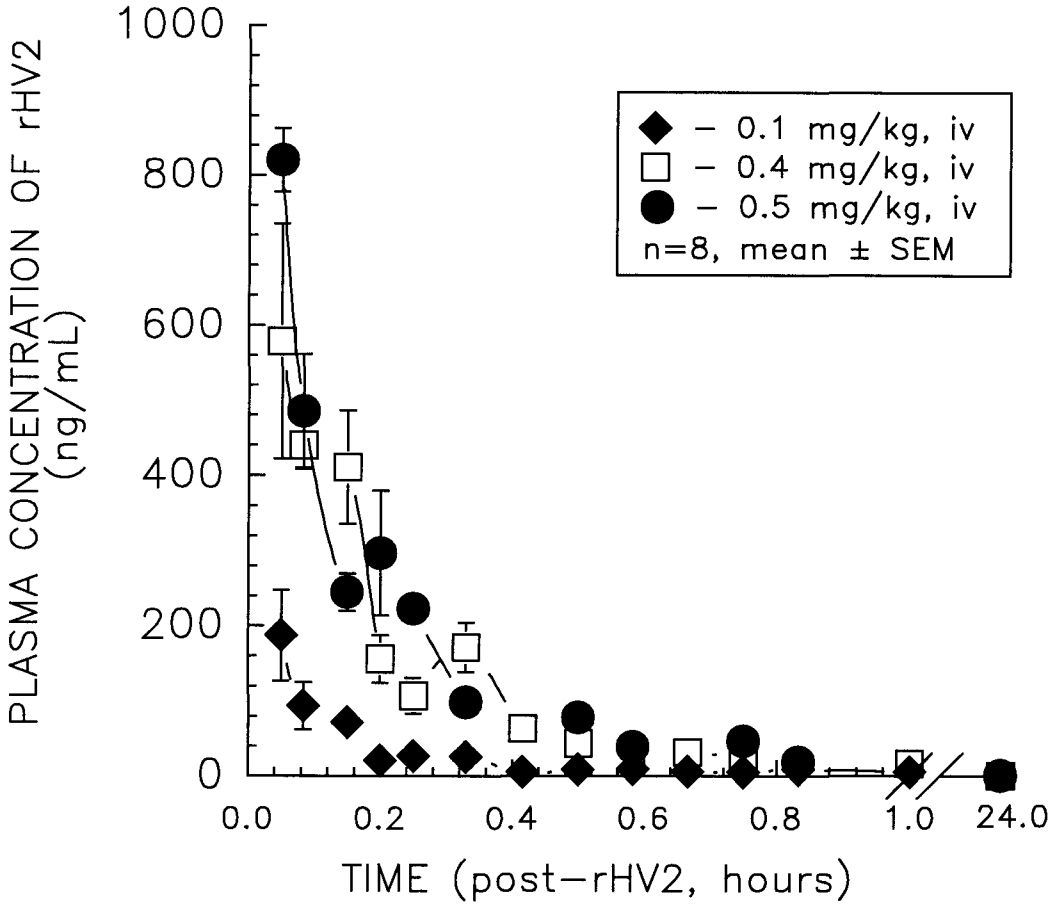


Figure 49. Pharmacokinetics of rHV2 after i.v. administration of increasing doses in rats. Male, Sprague-Dawley rats were administered with i.v. doses of 0.1 (♦), 0.4 (□) and 0.5 (●) mg/kg of rHV2 via tail vein. n = 8 rats were used in each treatment group. Plasma rHV2 concentrations were determined by competitive ELISA method. All data represent mean concentration from 8 rats ± SEM, each determined in duplicate.

($AUC_{0-\infty}$) was observed, i.e. ≈ 47, 134, and 165 ng-hr/mL, with increasing rHV2 doses. The areas under the first moment curves ($AUMC_{0-\infty}$) were approximately 36, 78 and 162 ng-hr^2/mL, respectively.

The mean residence times (MRT) were approximately 0.78, 0.59 and 0.98 hr after i.v. administration of 0.1, 0.4 and 0.5 mg/kg doses of rHV2, respectively, as listed in Table 8. No major variations were found in the terminal elimination rate constant (k_e) values between the three doses (2.28, 1.8 and 1.7 $hr.^{-1}$, respectively). Similarly, no major differences were found in the terminal elimination half-lives ($t_{1/2}$) after increasing doses. The $t_{1/2}$ values obtained were approximately 0.3, 0.39 and 0.42 hr, translating to 18, 23 and 25 minutes, respectively, after increasing i.v. doses of rHV2.

Both total clearance (Cl_{tot}) rates, as well as volume of distribution (V_d) values did not vary with increasing i.v. doses of rHV2. Cl_{tot} rates of 2.15, 3.0 and 3.0 L/hr/kg were obtained after administration of 0.1, 0.4 and 0.5 mg/kg, respectively. The volume of distribution ($V_{d)}$ values were 0.941, 1.7 and 1.8 L/kg, respectively, with corresponding escalating doses of rHV2 (Table 8).

A time course analysis of *ex vivo* antithrombin activity using the amidolytic anti-IIa activity was also performed in conjunction with pharmacokinetics of rHV2 in rats. Figure 50 shows the time course of thrombin inhibitory activity of rHV2 in rats after i.v. administration of 0.1, 0.4 and 0.5 mg/kg. Peak thrombin inhibitory activities were obtained at 3 min.

TABLE 8

PHARMACOKINETIC PARAMETERS OBTAINED AFTER SINGLE DOSE I.V. (0.1, 0.4 and 0.5 mg/kg) ADMINISTRATION OF rHV2 IN RATS

Parameter	0.1 mg/kg	0.4 mg/kg	0.5 mg/kg
C_{max} (ng/mL)	187.03	578.88	820.56
t_{max} (min)	3.00	3.00	3.00
k_e (hr^{-1})	2.28	1.79	1.66
$t_{1/2}$ (hr)	0.30	0.39	0.42
$AUC_{0-\infty}$ (ng-hr/mL)	46.60	133.83	164.70
$AUMC_{0-\infty}$ ($ng\text{-}hr^2/mL$)	36.35	78.32	162.26
MRT (hr)	0.78	0.59	0.98
Cl_{tot} (mL/hr/kg)	2145.80	2988.00	3030.00
V_d (mL/kg)	941.00	1669.00	1825.00

after administration of all three rHV2 doses. The maximum antithrombin effect observed was approximately 52, 81 and 97% after i.v. injection of 0.1, 0.4 and 0.5 mg/kg of rHV2. Hence, the antithrombin activity increased proportionally with increases in i.v. dose of rHV2.

The time course of *ex vivo* antithrombin activity closely followed the pharmacokinetics of rHV2 in rats. After each dose, the thrombin inhibitory activity declined quickly with very little residual activity after $\approx$ 45 minutes (Figure 50). The progression of antithrombin activity with time was related to the plasma concentrations of rHV2 obtained using the competitive ELISA method, after each i.v. dose of rHV2. The correlation coefficients were significant: 0.96, 0.96 and 0.90 for 0.1, 0.4 and 0.5 mg/kg dosages of rHV2, respectively.

S.C. Administration. rHV2 was administered in s.c. doses of 0.1, 0.5 and 1.0 mg/kg in the abdominal area of male, Sprague-Dawley rats, as described in the chapter on "Materials, Methods and Experimental protocols". n = 8 rats were used per treatment group and time schedule (composite data).

Figure 51 illustrates the plasma concentration-time curve after s.c. administration of rHV2 in rats. rHV2 was absorbed within the first 1 to 1.25 hours after administration of each dose. After the peak, rHV2 concentrations declined progressively as shown in Figure 51. Plasma concentrations

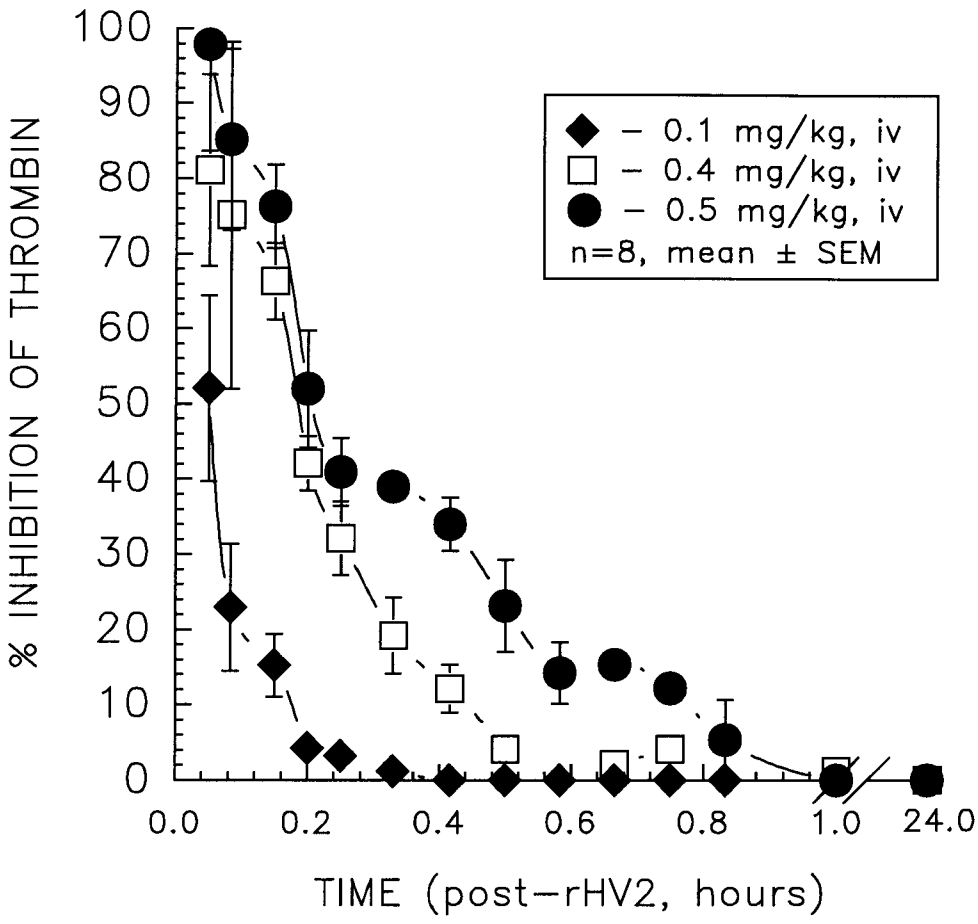


Figure 50. Time course of *ex vivo* antithrombin activity (chromogenic anti-IIa assay) of rHV2 in rats after i.v. administration. Male, Sprague-Dawley rats were administered with i.v. doses of 0.1 (◆), 0.4 (□) and 0.5 (●) mg/kg of rHV2 via tail vein. n = 8 rats were used in each treatment group. All data represent mean concentration from 8 rats ± SEM, each determined in duplicate.

obtained after each dose were proportional to the magnitude of the administered dose.

Table 9 lists the pharmacokinetic parameters after s.c. administration of rHV2 in rats. A t_{max} value of 1.25 hrs was obtained after administration of 0.1 and 0.5 mg/kg rHV2 doses, and that of 1 hr was obtained after administration of 1.0 mg/kg of rHV2. The peak plasma concentrations (C_{max}) increased linearly with increasing doses. The C_{max} values obtained were approximated to be 43, 272 and 547 ng/mL after administration of 0.1, 0.5 and 1.0 mg/kg, respectively. Similarly, dose dependent increases were observed in $AUC_{0-\infty}$ and $AUMC_{0-\infty}$ values. $AUC_{0-\infty}$ values obtained were $\approx$ 79, 581 and 1146 ng-hr/mL after administration of 0.1, 0.5 and 1.0 mg/kg rHV2 doses, respectively, as shown in Table 9. $AUMC_{0-\infty}$ values obtained were 112, 989 and 1947 ng-hr^2/ mL, for the corresponding doses.

The MRTs were calculated to be equal to 1.43, 1.70 and 1.70 hrs for the 0.1, 0.5 and 1.0 mg/kg doses, respectively (Table 9). There were no major differences between the three doses in k_e or $t_{1/2}$ values. The k_e values were 0.98, 1.02 and 1.15 hr^{-1} which corresponded to $t_{1/2}$ values of approximately 0.71, 0.68 and 0.60 hr (or 42, 41 and 36 minutes), respectively.

As depicted in Table 9, there were no major fluctuations in both Cl_{tot} rates as well as apparent V_d, after administration of increasing s.c. doses of rHV2. The Cl_{tot} rates were $\approx$ 255, 298 and 320 mL/kg/hr after

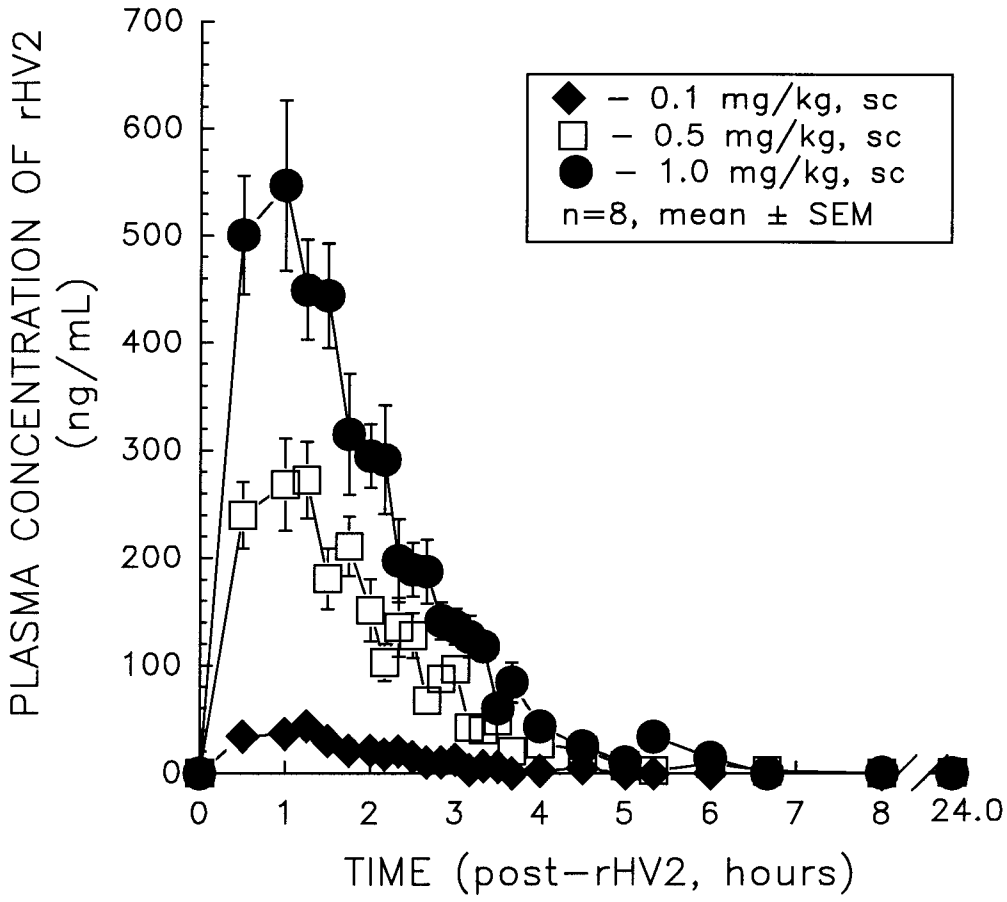


Figure 51. Pharmacokinetics of rHV2 after s.c. administration of increasing doses in rats. Male, Sprague-Dawley rats were administered with s.c.. doses of 0.1 (◆), 0.5 (□) and 1.0 (●) mg/kg of rHV2 in the abdominal area. n = 8 rats were used in each treatment group. Plasma rHV2 concentrations were determined by competitive ELISA method. All data represent mean concentration from 8 rats ± SEM, each determined in duplicate.

TABLE 9

PHARMACOKINETIC PARAMETERS OBTAINED AFTER SINGLE DOSE S.C. (0.1, 0.5 and 1.0 mg/kg) ADMINISTRATION OF rHV2 IN RATS

Parameter	0.1 mg/kg	0.5 mg/kg	1.0 mg/kg
C_{max} (ng/mL)	43.41	272.51	546.77
t_{max} (hr)	1.25	1.25	1.00
k_e (hr^{-1})	0.98	1.02	1.15
$t_{1/2}$ (hr)	0.71	0.68	0.60
$AUC_{0-\infty}$ (ng-hr/mL)	79.18	581.18	1146.54
$AUMC_{0-\infty}$ ($ng\text{-}hr^2/mL$)	112.84	989.21	1947.17
MRT (hr)	1.43	1.70	1.70
Cl_{tot} (mL/kg/hr)	255.11	298.54	320.09
V_d (mL/kg)	111.89	162.19	167.67

administration of 0.1, 0.5 and 1.0 mg/kg doses of rHV2. The corresponding apparent V_d values obtained were 112, 162 and 168 mL/kg, respectively.

The time course of *ex vivo* antithrombin activity was also studied after s.c. administration of rHV2, using the chromogenic anti-IIa assay. This progression after administration of all three s.c. doses of rHV2 is depicted in Figure 52. A dose dependent increase in thrombin inhibitory activity was observed after administration of each increasing dose of rHV2. Peak anti-IIa activity of approximately 16, 41 and 85% was observed at 1.5, 0.5 and 0.5 hrs after administration of 0.1, 0.5 and 1.0 mg/kg of rHV2, respectively.

The curves for the time course of *ex vivo* anti-IIa activity after s.c. administration (Figure 52), and the pharmacokinetic curves after s.c. administration (Figure 51) were similar. This was reflected by the significant correlation coefficients of 0.90 and 0.97 using the higher two dosages of rHV2, when the antithrombin activity was correlated with corresponding plasma rHV2 concentrations. In comparison, the correlation after the lowest dose, i.e. 0.1 mg/kg, s.c., was poor ($r = 0.78$).

5. Evaluation of Renal Function after rHV2 Administration in Rats

A single dose ^{3}H-inulin clearance study was performed to evaluate renal function after i.v. and s.c. administration of rHV2 in rats. rHV2 was used in an i.v. dose of 0.5 mg/kg and s.c. dose of 1.0 mg/kg. 24 hours after rHV2 administration, ^{3}H-inulin was injected intravenously at a dose of 10 μCi

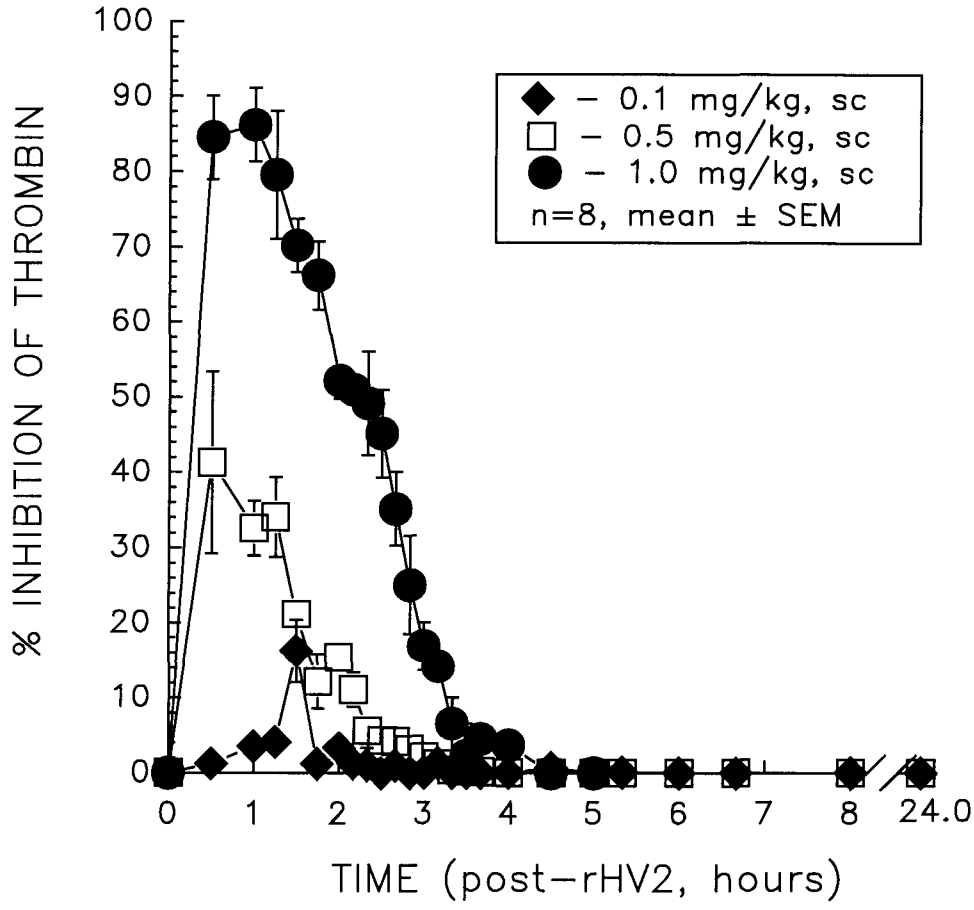


Figure 52. Time course of *ex vivo* antithrombin activity (chromogenic anti-IIa assay) of rHV2 in rats after s.c. administration. Male, Sprague-Dawley rats were administered with s.c.. doses of 0.1 (◆), 0.5 (□) and 1.0 (●) mg/kg of rHV2 in the abdominal area. n = 8 rats were used in each treatment group. All data represent mean concentration from 8 rats ± SEM, each determined in duplicate.

per rat.

Figure 53 illustrates the disposition of ^{3}H-inulin in rats after i.v. and s.c. administration of rHV2, along with appropriate saline treated controls. The dpm/mL obtained in each sample using a liquid scintillation counter were converted to μCi/mL and plotted on a log scale vs. circulation time of ^{3}H-inulin. Table 10 lists the corresponding dispositional parameters of ^{3}H-inulin, using non-compartmental methods. The dispositional curves of ^{3}H-inulin after rHV2 and corresponding saline treatment (i.v. or s.c.) were closely parallel to one other, as shown in Figure 53.

The rate of ^{3}H-inulin clearance (Cl_{in}) was calculated in each rHV2 treated group and compared with corresponding saline treated group. Cl_{in} was calculated using the formula, Dose/$AUC_{0-\infty}$. Cl_{in} was equal to 1.30 ± 0.11 mL/min/100g body weight (saline) vs. 1.10 ± 0.13 mL/min/100g body weight (rHV2) after i.v. administration, and 1.46 ± 0.12 mL/min/100g body weight (saline) vs. 1.36 ± 0.14 mL/min/100g body weight (rHV2) after s.c. administration (Table 10). There were no significant differences in Cl_{in} values between all four treatments ($p = 0.132$, one-way ANOVA). The calculated Cl_{in} values closely matched the reported value for glomerular filtration rate (GFR) of 1.02 mL/min/100g body weight in rats (Ringler and Dabich, 1979).

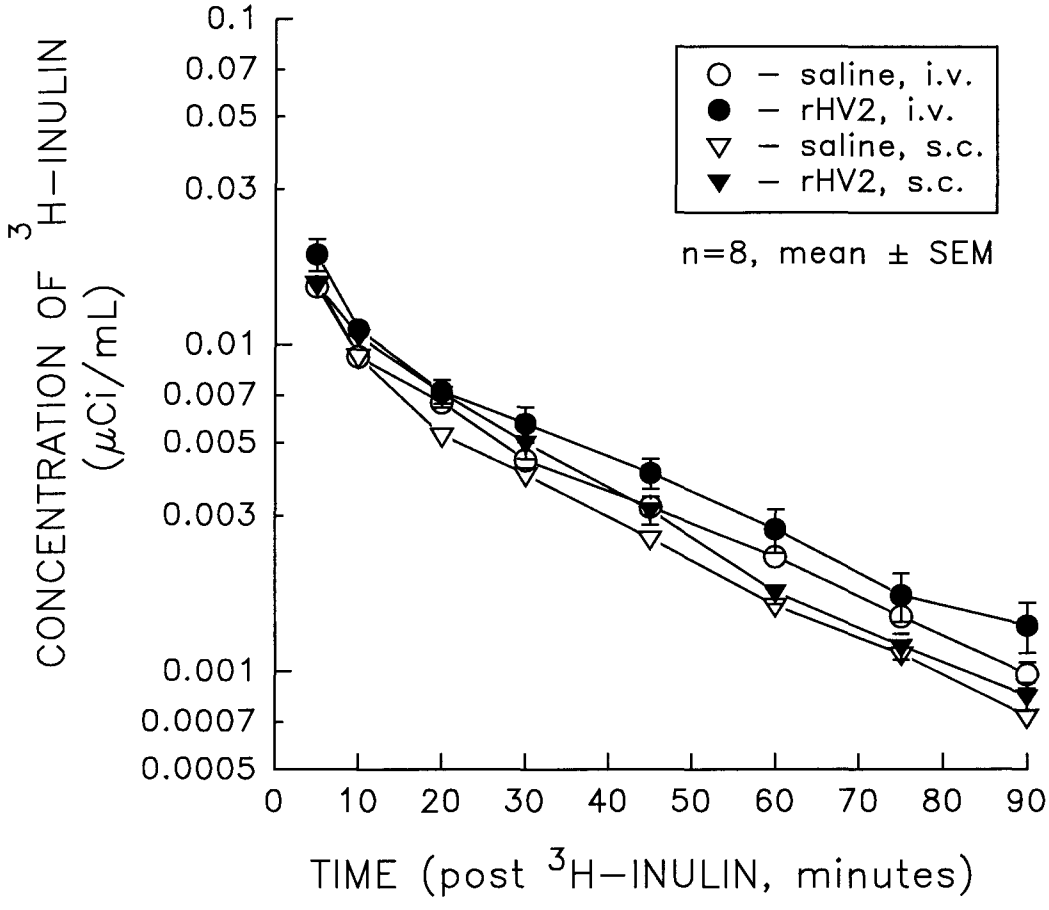


Figure 53. Plasma concentration-time curve of ^{3}H-inulin in rats pretreated with rHV2 compared with saline treated controls. Male, Sprague-Dawley rats were pretreated with i.v. bolus (0.5 mg/kg) or s.c. (1.0 mg/kg) of rHV2 along with saline treated controls. At 24 hours, a single dose ^{3}H-inulin (10 μCi) clearance study was performed.

Symbols indicate i.v. saline (○), i.v. rHV2 (●), s.c. saline (▽) and s.c. rHV2 (▼) administration. All data represent the mean concentration in 8 rats, each determined in triplicate, ± SEM.

TABLE 10

DISPOSITIONAL PARAMETERS OBTAINED IN ^{3}H-INULIN CLEARANCE STUDIES IN RATS

Parameter	Saline, i.v.	rHV2, i.v.	Saline, s.c.	rHV2, s.c.
Cl_{in} (mL/min/100g)	1.30 ± 0.11	1.10 ± 0.13	1.46 ± 0.12	1.36 ± 0.14

No significant differences in Cl_{in} between treatment groups (one-way ANOVA). All data represent the mean of individual parameters from 8 rats ± SEM.

5a. Serum Chemistry Profile in Rats after Administration of rHV2

Male, Sprague-Dawley rats (n=8 per treatment group) were administered with 0.5 mg/kg and 1.0 mg/kg of rHV2 via i.v. and s.c. routes, respectively. Serum chemistry parameters were determined prior to (baseline) and 24 hours after treatment with rHV2, and compared with saline treated control groups.

I.V. Administration. The results from serum chemistry tests after i.v. administration of rHV2 are listed in Tables 11 and 12. rHV2 treatment led to a significant decrease in triglyceride level at 24 hours (66 ± 8 mg/dL) from corresponding baseline value (116 ± 18 mg/dL) {$p<0.05$, paired t-test, Table 11}. In the saline treated groups, a significant decrease was found in

TABLE 11

SERUM CHEMISTRY PROFILE IN RATS AFTER I.V. ADMINISTRATION (0.5 mg/kg) OF rHV2 (part I)

Test	Saline Treatment (iv)		rHV2 Treatment (iv)	
	Baseline	24 hours	Baseline	24 hours
ALB (g/dL)	2.5 ± 0.1	2.4 ± 0.1	2.6 ± 0.1	2.4 ± 0.1
T PROT (g/dL)	6.3 ± 0.2	6.1 ± 0.1	6.3 ± 0.1	6.2 ± 0.0
T BIL (mg/dL)	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.2 ± 0.0
ALP (IU/L)	178 ± 8	161 ± 9	199 ± 14	194 ± 13
AST (IU/L)	142 ± 16	125 ± 11	120 ± 9	99 ± 8
ALT (IU/L)	57 ± 3	50 ± 3	58 ± 3	60 ± 3
γ-GT (IU/L)	1.4 ± 0.8	**0.5 ± 0.2	1.0 ± 0.5	0.3 ± 0.3
CHOL (mg/dL)	87 ± 5	86 ± 6	95 ± 4	95 ± 3
TRIG (mg/dL)	130 ± 10	*75 ± 7	116 ± 18	*66 ± 8
GLU (mg/dL)	137 ± 15	127 ± 7	138 ± 8	125 ± 7

*-$p<0.05$, **-$p<0.10$ - significant difference from corresponding baseline (paired t-test). All data represent the mean observation from 6 to 8 rats ± SEM. See List of Abbreviations for explanation of terms.

TABLE 12

SERUM CHEMISTRY PROFILE IN RATS AFTER I.V. ADMINISTRATION (0.5 mg/kg) OF rHV2 (part II)

Test	Saline Treatment (iv)		rHV2 Treatment (iv)	
	Baseline	24 hours	Baseline	24 hours
LDH (IU/L)	2363 ± 531	**1925 ± 259	1501 ± 269	1693 ± 445
BUN (mg/dL)	21 ± 2	20 ± 1	21 ± 1	20 ± 1
CREAT (mg/dL)	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	0.5 ± 0.1
U ACID (mg/dL)	1.6 ± 0.2	1.4 ± 0.1	1.4 ± 0.1	1.3 ± 0.1
Na^+ (mmol/L)	145 ± 1	147 ± 1	146 ± 11	134 ± 8
K^+ (mmol/L)	6.4 ± 0.3	6.0 ± 0.1	5.3 ± 0.3	5.3 ± 0.4
Cl^- (mmol/L)	102 ± 1	103 ± 1	94 ± 4	92 ± 6
CO_2 (mmol/L)	22 ± 2	24 ± 1	22 ± 1	*27 ± 1
Ca^{+2} (mg/dL)	10.5 ± 0.2	10.4 ± 0.1	10.4 ± 0.1	10.4 ± 0.1
PHOS (mg/dL)	9.3 ± 0.9	9.0 ± 0.3	8.3 ± 0.3	8.5 ± 0.2

*-$p<0.05$, **-$p<0.10$ - significant difference from corresponding baseline (paired t-test). All data represent the mean observation from 6 to 8 rats ± SEM. See List of Abbreviations for explanation of terms.

triglyceride (TRIG), lactic dehydrogenase (LDH) and gamma-glutamyl transferase (γ-GT) levels at 24 hours when compared with baseline levels (Tables 11 and 12) {paired t-test}. There was a significant increase in total carbon dioxide (CO_2) levels in rHV2 treated groups at 24 hours (27 ± 1 mmol/L) when compared with corresponding baseline value (22 ± 1 mmol/L) {$p < 0.05$, paired t-test, Table 12}. However, there was no significant change in CO_2 level between saline treatment and rHV2 treatment (independent t-test) at baseline and 24 hours.

It is interesting to note that no significant differences were seen in most serum chemistry parameters between saline and rHV2 treated groups both at baseline and 24 hours (independent t-test) after i.v. administration of rHV2.

S.C. Administration. The serum chemistry parameters obtained before and after s.c. administration (1.0 mg/kg) of rHV2 are listed in Tables 13 and 14. As observed with the i.v. administration, there was a significant decrease in triglyceride level at 24 hours (74 ± 8 mg/dL) after rHV2 administration, when compared with corresponding baseline (126 ± 11 mg/dL) {$p < 0.05$, paired t-test, Table 13}. A significant decrease in LDH level was observed at 24 hours (1546 ± 251 IU/L) after rHV2 administration when compared with corresponding baseline (2279 ± 350 IU/L) {$p < 0.05$, paired t-test, Table 14}.

TABLE 13

SERUM CHEMISTRY PROFILE IN RATS AFTER S.C. ADMINISTRATION (1.0 mg/kg) OF rHV2 (part I)

Test	Saline Treatment (sc)		rHV2 Treatment (sc)	
	Baseline	24 hours	Baseline	24 hours
ALB (g/dL)	2.4 ± 0.1	*2.6 ± 0.1	2.4 ± 0.1	2.4 ± 0.1
T PROT (g/dL)	6.0 ± 0.1	6.3 ± 0.2	6.1 ± 0.2	6.0 ± 0.1
T BIL (mg/dL)	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.1
ALP (IU/L)	200 ± 11	181 ± 7	205 ± 14	187 ± 12
AST (IU/L)	107 ± 13	146 ± 32	127 ± 9	117 ± 9
ALT (IU/L)	53 ± 8	58 ± 14	53 ± 2	50 ± 3
γ-GT (IU/L)	1.8 ± 0.7	1.3 ± 0.5	1.4 ± 0.5	1.1 ± 0.5
CHOL (mg/dL)	93 ± 7	97 ± 7	91 ± 6	97 ± 8
TRIG (mg/dL)	114 ± 11	92 ± 9	126 ± 11	*74 ± 8
GLU (mg/dL)	154 ± 6	146 ± 8	131 ± 4	132 ± 13

*-$p < 0.05$ - significant difference from corresponding baseline (paired t-test). All data represent the mean observation from 6 to 8 rats ± SEM. See List of Abbreviations for explanation of terms.

TABLE 14

SERUM CHEMISTRY PROFILE IN RATS AFTER S.C. ADMINISTRATION (1.0 mg/kg) OF rHV2 (part II)

Test	Saline Treatment (sc)		rHV2 Treatment (sc)	
	Baseline	24 hours	Baseline	24 hours
LDH (IU/L)	1281 ± 209	1680 ± 415	2279 ± 350	*1546 ± 251
BUN (mg/dL)	19 ± 2	22 ± 2	21 ± 1	22 ± 1
CREAT (mg/dL)	0.6 ± 0.1	0.6 ± 0.0	0.7 ± 0.1	0.6 ± 0.0
U ACID (mg/dL)	1.3 ± 0.1	1.4 ± 0.1	1.6 ± 0.1	1.3 ± 0.1
Na^+ (mmol/L)	144 ± 1	151 ± 5	146 ± 0	142 ± 4
K^+ (mmol/L)	6.0 ± 0.3	5.9 ± 0.3	5.9 ± 0.2	5.4 ± 0.2
Cl^- (mmol/L)	102 ± 1	106 ± 3	101 ± 0	99 ± 3
CO_2 (mmol/L)	20 ± 2	22 ± 2	24 ± 1	23 ± 1
Ca^{+2} (mg/dL)	10.0 ± 0.6	10.7 ± 0.3	10.4 ± 0.2	10.3 ± 0.1
PHOS (mg/dL)	8.2 ± 0.6	8.7 ± 0.1	8.7 ± 0.3	8.6 ± 0.2

*-$p < 0.05$ - significant difference from corresponding baseline (paired t-test). All data represent the mean observation from 6 to 8 rats ± SEM. See List of Abbreviations for explanation of terms.

No significant differences were found in most serum chemistry parameters between saline and rHV2 treatments both at baseline and 24 hours (independent t-test).

5b. Hematological Profile in Rats after Administration of rHV2

I.V. Administration. Table 15 lists the hematological parameters prior to, and after (24 hours) i.v. dosing (0.5 mg/kg) of rHV2 in rats when compared with saline treatment. A slight, yet significant ($p<0.1$, paired t-test) decrease in RBC count, hemoglobin (HGB) level and hematocrit (HCT) was observed at 24 hours after saline treatment, when compared with corresponding baseline. A significant decrease was also noted in the above parameters at 24 hours after rHV2 treatment, when compared to corresponding baseline (Table 15). In the rHV2 treated group, a significant decrease was seen at 24 hours in platelet count ($818 \pm 34 \times 10^3/\mu L$) compared to baseline ($925 \pm 27 \times 10^3/\mu L$) {$p<0.05$, paired t-test}. No significant changes were seen in most hematological markers between saline and rHV2 treatments at both baseline and 24 hours, as shown in Table 15 (independent t-test).

S.C. Administration. The hematological profile after s.c. rHV2 treatment (1.0 mg/kg) in rats is listed in Table 16. A significant decrease ($p<0.05$, paired t-test) was noted in RBC count, HGB level and HCT value at 24 hours after rHV2 treatment compared to baseline values. A slight

decrease in HCT was also seen 24 hours after saline treatment ($p<0.1$, paired t-test) as shown in Table 16. No significant differences were found in most hematological parameters between saline and rHV2 treatments at baseline and 24 hours (independent t-test).

TABLE 15

HEMATOLOGICAL PROFILE IN RATS AFTER I.V. ADMINISTRATION (0.5 mg/kg) OF rHV2

Test	Saline Treatment (iv)		rHV2 Treatment (iv)	
	Baseline	24 hours	Baseline	24 hours
WBC ($\times 10^3/\mu L$)	15.6 ± 1.3	15.6 ± 0.9	13.8 ± 0.6	14.1 ± 1.3
RBC ($\times 10^6/\mu L$)	8.1 ± 0.2	**7.4 ± 0.1	7.7 ± 0.2	**7.3 ± 0.2
HGB (g/dL)	16.6 ± 0.4	**15.6 ± 0.2	15.8 ± 0.3	*14.9 ± 0.3
HCT (%)	47.6 ± 1.5	**43.0 ± 0.8	44.1 ± 0.8	*41.6 ± 0.8
MCV (fL)	58.4 ± 0.5	57.9 ± 0.4	57.0 ± 0.6	57.1 ± 0.5
MCH (pg)	20.4 ± 0.3	20.9 ± 0.2	20.4 ± 0.3	20.4 ± 0.3
MCHC (g/dL)	35.0 ± 0.7	36.2 ± 0.3	35.8 ± 0.6	35.8 ± 0.4
RDW (%)	11.7 ± 0.2	11.5 ± 0.1	11.5 ± 0.2	11.3 ± 0.2
Platelet ($\times 10^3/\mu L$)	944 ± 55	944 ± 97	926 ± 27	*818 ± 34
MPV (fL)	6.0 ± 0.2	5.7 ± 0.1	5.6 ± 0.1	5.7 ± 0.1

*-$p<0.05$, **-$p<0.10$ - significant difference from corresponding baseline. All data represent the mean observation from 6 to 8 rats ± SEM. See List of Abbreviations for explanation of terms.

TABLE 16

HEMATOLOGICAL PROFILE IN RATS AFTER S.C. ADMINISTRATION (1.0 mg/kg) OF rHV2

Test	Saline Treatment (sc)		rHV2 Treatment (sc)	
	Baseline	24 hours	Baseline	24 hours
WBC ($\times 10^3/\mu L$)	12.4 ± 0.9	13.5 ± 1.1	13.8 ± 0.4	14.4 ± 0.6
RBC ($\times 10^6/\mu L$)	7.7 ± 0.2	7.2 ± 0.2	7.6 ± 0.2	*7.0 ± 0.2
HGB (g/dL)	15.3 ± 0.8	15.0 ± 0.4	15.8 ± 0.3	*14.6 ± 0.3
HCT (%)	44.1 ± 1.0	**41.5 ± 1.0	43.6 ± 1.0	*40.2 ± 0.7
MCV (fL)	57.5 ± 0.2	57.4 ± 0.4	57.6 ± 0.9	57.6 ± 1.0
MCH (pg)	20.0 ± 0.8	20.7 ± 0.1	20.8 ± 0.3	20.9 ± 0.3
MCHC (g/dL)	34.6 ± 1.3	36.1 ± 0.2	36.2 ± 0.2	36.4 ± 0.4
RDW (%)	11.2 ± 0.2	11.4 ± 0.1	11.6 ± 0.1	11.6 ± 0.1
Platelet ($\times 10^3/\mu L$)	1027 ± 51	1049 ± 44	1011 ± 38	904 ± 41
MPV (fL)	5.9 ± 0.2	5.8 ± 0.2	5.8 ± 0.2	5.8 ± 0.2

*-$p<0.05$, **-$p<0.10$ - significant difference from corresponding baseline. All data represent the mean observation from 6 to 8 rats ± SEM. See List of Abbreviations for explanation of terms.

6. Investigation of Pharmacokinetics and Pharmacodynamics of rHV2 after Single Dose Administration in Dogs

Male, mongrel dogs (25-30 kg) were administered with increasing i.v. and s.c. doses of rHV2 in a parallel fashion, as described in the chapter on "Materials, Methods and Experimental Protocols". rHV2 was used in i.v. and s.c. doses of 0.25, 0.5 and 1.0 mg/kg.

I.V. Administration. The plasma samples obtained after rHV2 administration in dogs at various time periods were analyzed using coagulation tests, such as APTT (OT), APTT (Dade), TT (20 U/mL), Ca^{+2}TT (10 U/mL) and Ca^{+2}TT (20 U/mL), as well as the amidolytic anti-IIa assay.

The pharmacokinetics after i.v. administration of rHV2 is depicted in Figure 54. As observed in the rat and the rabbit studies, peak plasma concentrations were obtained at 3 minutes (t_{max}), followed by a rapid decline, reaching $\approx$ 1 to 2 μg/mL at 60 to 120 minutes after drug administration. The pharmacokinetic parameters obtained after single dose administration of rHV2 in all three doses, are shown in Table 17. Peak plasma rHV2 concentrations were 3.51, 5.40 and 7.16 μg/mL after administration of 0.25, 0.5 and 1.0 mg/mL, respectively. The corresponding $AUC_{0-\infty}$ values were 1.49, 3.17 and 6.34 μg-hr/mL, respectively. Dose dependent, though non-linear, increases were found between the three doses in the C_{max} , $AUC_{0-\infty}$ and $AUMC_{0-\infty}$ values.

The k_e values after escalating i.v. doses of rHV2 in dogs were 0.65,

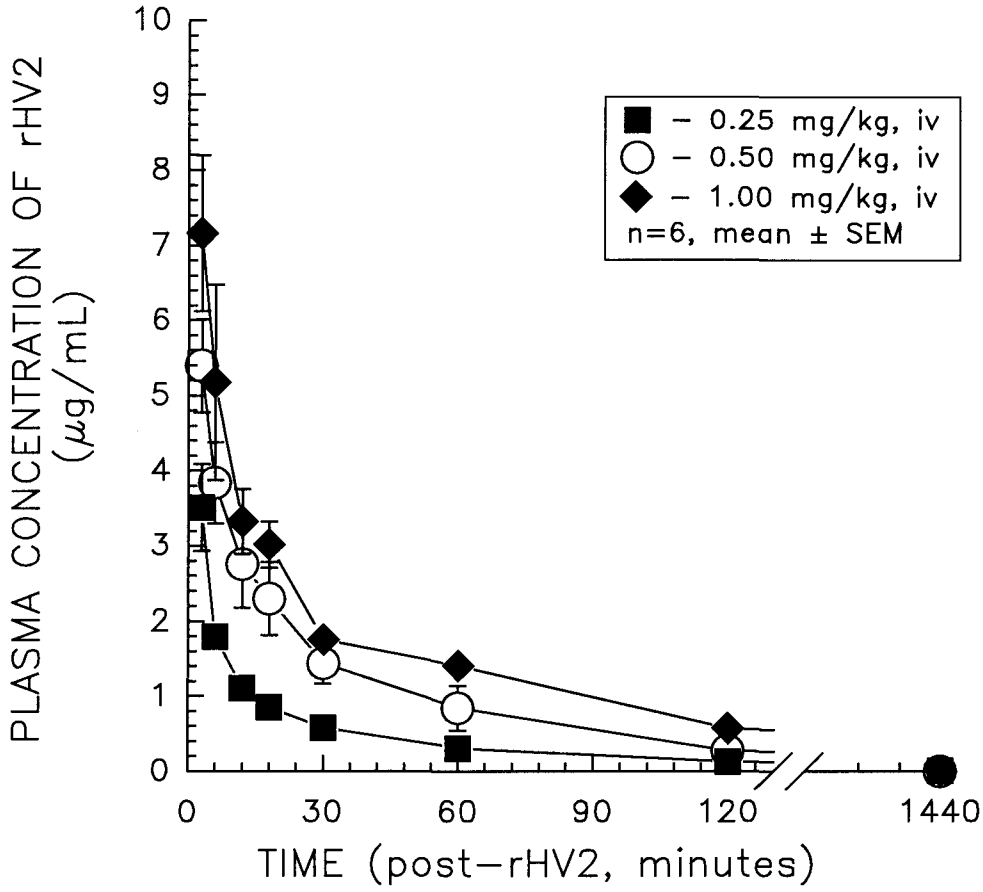


Figure 54. Pharmacokinetics of rHV2 in dogs after escalating i.v. bolus administration. Plasma concentrations were determined using competitive ELISA method. rHV2 was administered in i.v. doses of 0.25 (■), 0.5 (○) and 1.0 (◆) mg/kg. All data represent the mean concentration from 6 dogs ± SEM, each determined in duplicate.

0.85 and 0.74 hr^{-1}, respectively. The corresponding $t_{1/2}$ values obtained were 1.07, 0.80 and 0.94 hr (translating to 64, 48 and 56 minutes), respectively (Table 17). The MRTs after escalating i.v. doses of rHV2 were 1.03, 1.14 and 1.46 hrs., respectively. The Cl_{tot} rates and V_d were 167, 158 and 156 mL/hr/kg, and 256, 194 and 212 mL/kg, after i.v. administration of 0.25, 0.50 and 1.0 mg/kg, respectively. No major differences were found in the $t_{1/2}$, k_e, Cl_{tot}, and V_d values between the three doses of rHV2, after i.v. administration in dogs (Table 17).

The results from *ex vivo* coagulation tests, obtained after i.v. administration of rHV2 are described in Figures 55 to 59. The time course of *ex vivo* APTT (OT) response after administration of all three doses of rHV2 is represented in Figure 55. The response is expressed as a mean percent increase from corresponding baseline value from six dogs. The baseline APTT (OT) obtained was about 16 seconds. A dose dependent peak increase in APTT (OT) was obtained at 3 minutes after rHV2 administration in all three dosing groups. Thereafter, the responses decreased steadily to baseline values between 60 and 120 minutes. Similarly, a dose dependent peak increase in clotting time was observed in the case of APTT (Dade), as shown in Figure 56. The baseline APTT (Dade) was about 12 seconds. Again, there was a sharp decline in response with time, reaching close to baseline values at about 120 minutes.

The time course of TT (20 U/mL) after step-wise augmentation of rHV2

TABLE 17

PHARMACOKINETIC PARAMETERS OBTAINED AFTER SINGLE DOSE I.V. (0.25, 0.50 and 1.0 mg/kg) ADMINISTRATION OF rHV2 IN DOGS

Parameter	0.25 mg/kg	0.50 mg/kg	1.0 mg/kg
C_{max} (μg/mL)	3.51	5.40	7.16
t_{max} (min)	3.00	3.00	3.00
k_e (hr^{-1})	0.65	0.85	0.74
$t_{1/2}$ (hr)	1.07	0.80	0.94
$AUC_{0-\infty}$ (μg-hr/mL)	1.49	3.17	6.34
$AUMC_{0-\infty}$ (μg-hr^2/mL)	1.53	3.60	9.36
MRT (hr)	1.03	1.14	1.46
Cl_{tot} (mL/hr/kg)	167.00	158.00	156.00
V_d (mL/kg)	256.00	194.00	212.39

doses is depicted in Figure 57. The baseline TT (20 U/mL) was about 8 seconds. Three minutes after rHV2 administration, the clotting times, measured by TT (20 U/mL) increased to > 300 seconds (> 40-fold increase from corresponding baseline values) in each treatment group. This increase remained sustained up to 45 minutes after doses of 0.25 and 0.5 mg/kg, and up to 60 minutes after a dose of 1.0 mg/kg, respectively. The responses returned to baseline values at about 120 minutes in all three treatment groups.

The changes in Ca^{+2}TT (10 U/mL) and Ca^{+2}TT (20 U/mL) after rHV2 administration in escalating doses are depicted in Figures 58 and 59, respectively. The baseline Ca^{+2}TT (10 U/mL) and Ca^{+2}TT (20 U/mL) were about 11 and 6 seconds, respectively. In the case of Ca^{+2}TT (10 U/mL), there was a sustained, maximum 30- to 40-fold increase in clotting times at 3 minutes (x 300 seconds in measured clotting time) after rHV2 administration in the range of doses employed. The Ca^{+2}TT (10 U/mL) values remained steady at the maximal response for up to 18, 30 and 60 minutes after administration of 0.25, 0.5 and 1.0 mg/kg, respectively. It was interesting to note that at the higher two treatment doses, a 10-fold (0.5 mg/kg) and 25-fold increase (1.0 mg/kg) in response persisted even at 120 minutes.

Similarly, Ca^{+2}TT (20 U/mL) responses increased in a dose dependent fashion, as described in Figure 59. At 3 minutes, there was almost a 50-fold increase in Ca^{+2}TT (20 U/mL) with the lowest dose, and a 60-fold increase in response with the higher two doses of rHV2. In the 0.25 mg/kg treatment

group, the response remained at the maximum level for about 6 minutes, after which it decreased progressively until it reached baseline values at 60 minutes. The higher two treatment groups exhibited maximal responses for up to 18 minutes, after which the intensity of responses decreased steadily in a dose dependent manner. In comparison with $Ca^{+2}TT$ (10 U/mL), the clotting response using $Ca^{+2}TT$ (20 U/mL) decreased to almost zero at 120 minutes after rHV2 administration of 0.5 mg/kg.

The *ex vivo* antithrombin activity as measured by the amidolytic anti-IIa assay is illustrated in Figure 60. The highest *ex vivo* antithrombin activity was observed at 3 minutes with about 90% thrombin inhibition for the 0.25 mg/kg treatment group, and an almost 100% thrombin inhibition for the higher two doses of rHV2. The thrombin inhibitory activity after the lower two doses decreased more rapidly, in a parallel fashion. After administration of 1.0 mg/kg, the activity remained at x 90% for up to 18 minutes followed by a slower progression toward baseline. It is interesting to note that a 40% and 20% thrombin inhibitory activity persisted with the highest dose at 60 and 120 minutes, respectively (Figure 60).

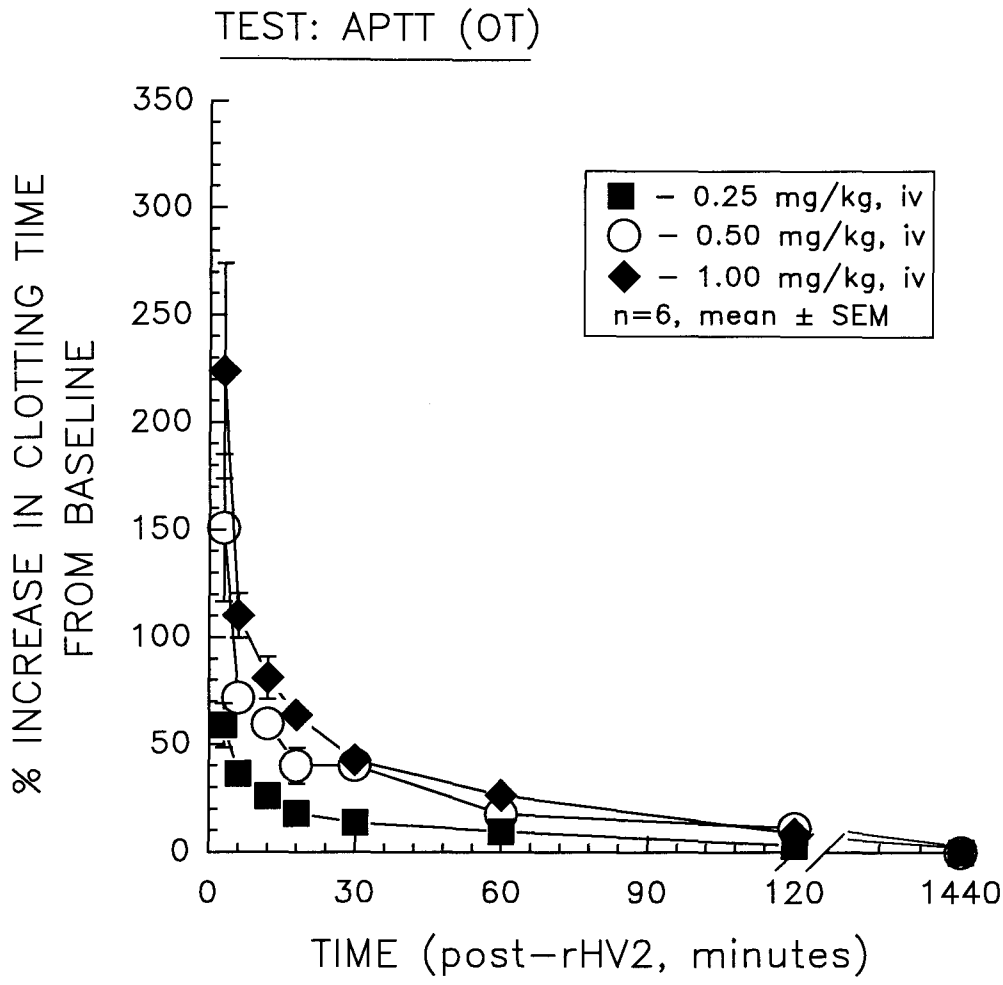


Figure 55. Time-course of *ex vivo* antithrombin activity [APTT (OT)] of rHV2, after administration increasing i.v. doses in dogs. rHV2 was used in doses of 0.25 (■), 0.5 (○) and 1.0 (◆) mg/kg. All data represent the mean concentration from 6 dogs ± SEM, each determined in duplicate.

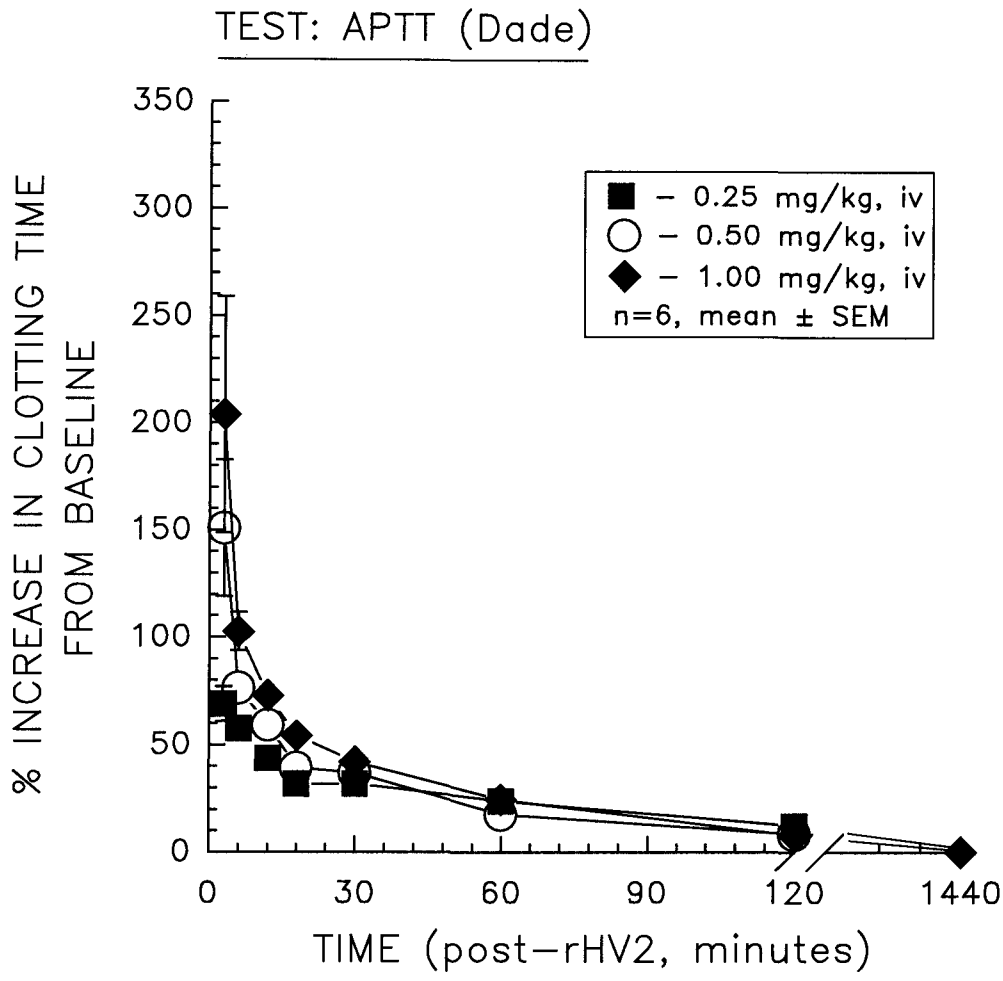


Figure 56. Time-course of *ex vivo* antithrombin activity [APTT (Dade)] of rHV2 after administration increasing i.v. doses in dogs. rHV2 was used in doses of 0.25 (■), 0.5 (○) and 1.0 (◆) mg/kg. All data represent the mean concentration from 6 dogs ± SEM, each determined in duplicate.

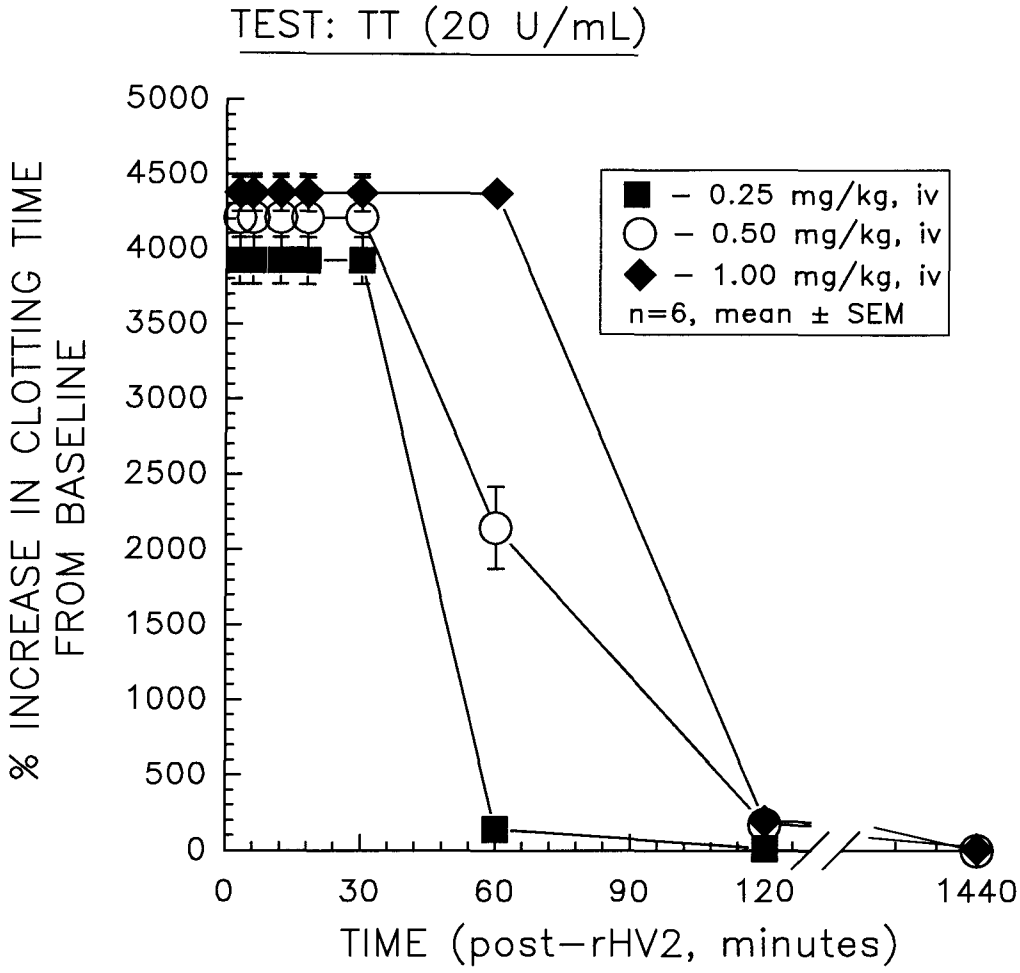


Figure 57. Time-course of *ex vivo* antithrombin activity TT (20 U/mL) of rHV2 after administration increasing i.v. doses in dogs. rHV2 was used in doses of 0.25 (■), 0.5 (○) and 1.0 (◆) mg/kg. All data represent the mean concentration from 6 dogs ± SEM, each determined in duplicate.

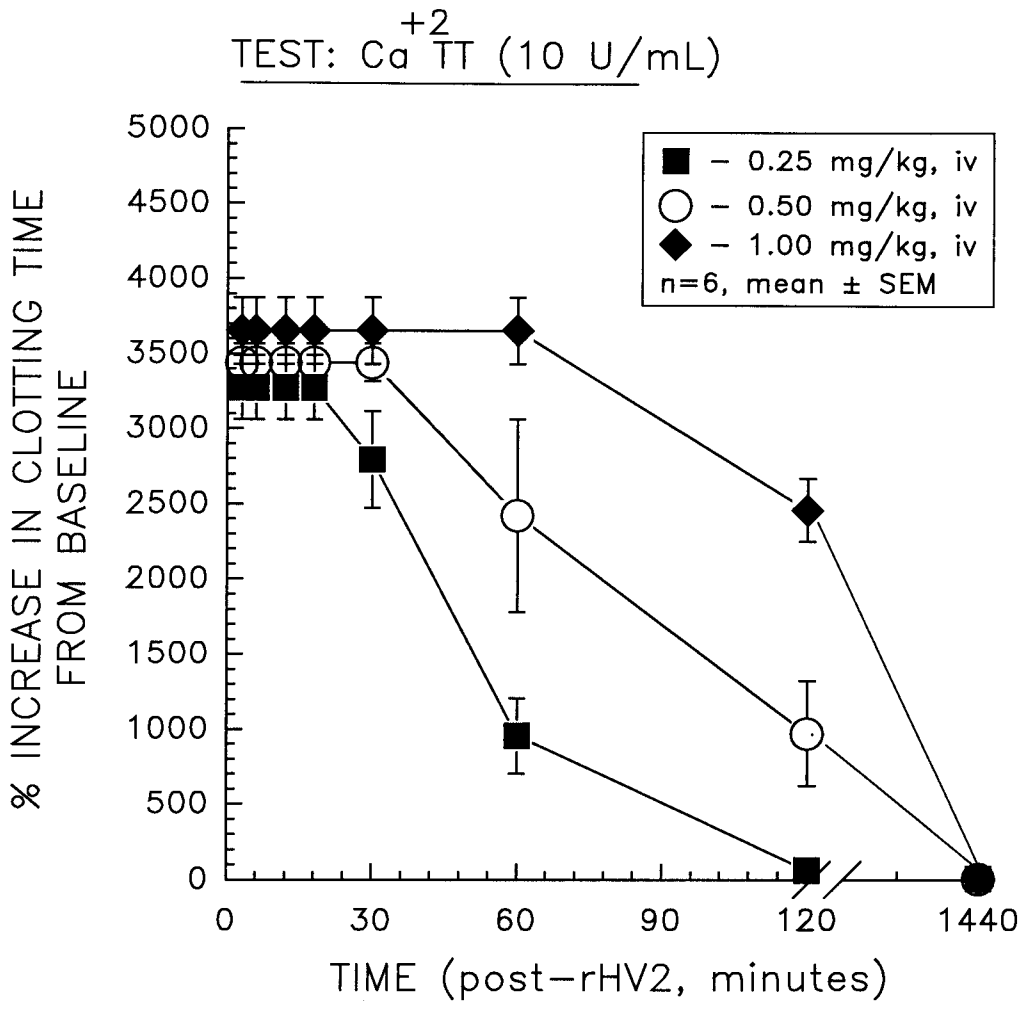


Figure 58. Time-course of *ex vivo* antithrombin activity $Ca^{+2}TT$ (10 U/mL) of rHV2 after administration increasing i.v. doses in dogs. rHV2 was used in doses of 0.25 (■), 0.5 (○) and 1.0 (◆) mg/kg. All data represent the mean concentration from 6 dogs ± SEM, each determined in duplicate.

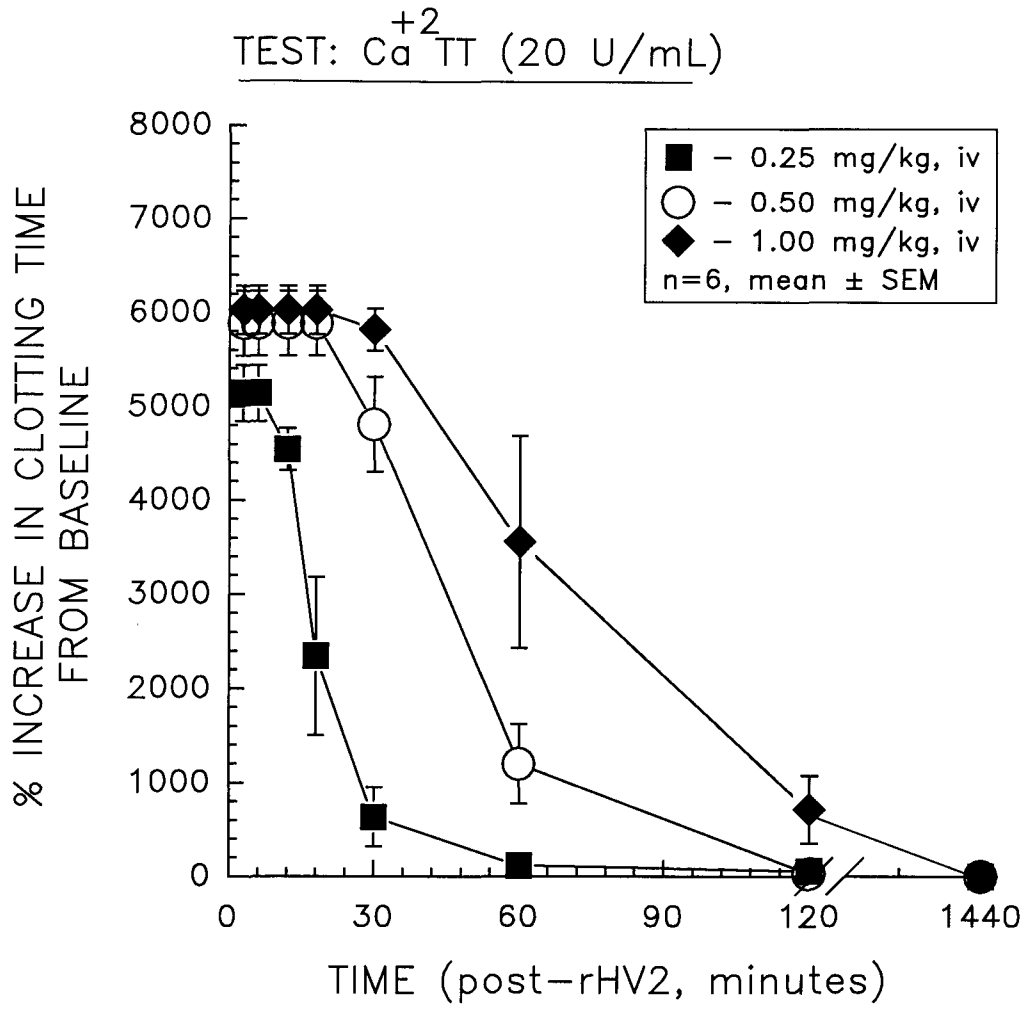


Figure 59. Time-course of *ex vivo* antithrombin activity $Ca^{+2}TT$ (20 U/mL) of rHV2 after administration increasing i.v. doses in dogs. rHV2 was used in doses of 0.25 (■), 0.5 (○) and 1.0 (◆) mg/kg. All data represent the mean concentration from 6 dogs ± SEM, each determined in duplicate.

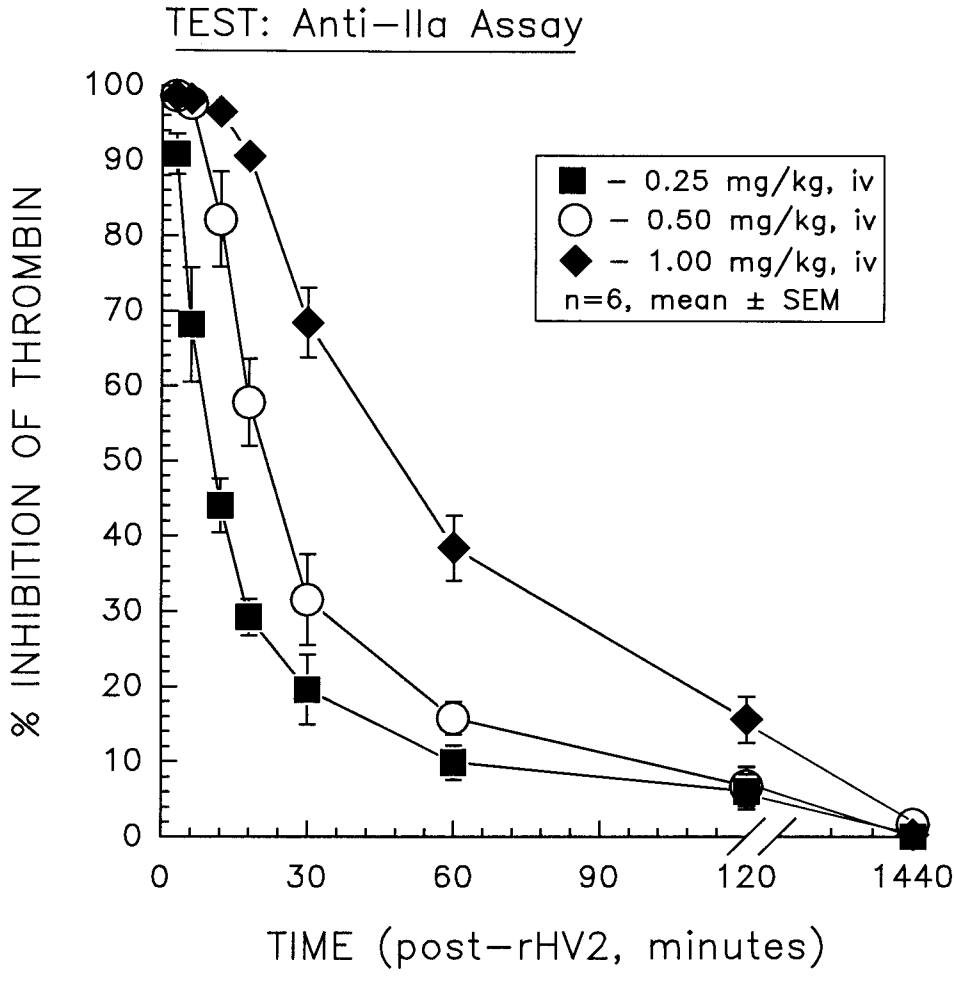


Figure 60. Time-course of *ex vivo* antithrombin activity of rHV2 after administration increasing i.v. doses in dogs (chromogenic anti-IIa assay). rHV2 was used in doses of 0.25 (■), 0.5 (○) and 1.0 (◆) mg/kg. All data represent the mean concentration from 6 dogs ± SEM, each determined in duplicate.

S.C. Administration. The plasma samples obtained after rHV2 administration were screened for *ex vivo* antithrombin activity using coagulation tests, ELISA and amidolytic anti-IIa assay.

Figure 61 illustrates the pharmacokinetics after single dose s.c. administration of rHV2 in doses of 0.25, 0.50 and 1.0 mg/kg. Table 18 lists the pharmacokinetic parameters of rHV2 after escalating s.c. doses. As seen in the i.v. studies, there was a dose dependent increase in peak plasma concentrations. The C_{max} values obtained were 0.43, 0.80 and 1.07 μg/mL, after administration of 0.25, 0.5 and 1.0 mg/kg, respectively. The corresponding t_{max} values were 90, 120 and 90 minutes, respectively. There were dose dependent increases in $AUC_{0-\infty}$, $AUMC_{0-\infty}$ and C_{max} values between the three doses (Table 18).

As observed in the i.v. studies in dogs, no major differences were obtained in Cl_{tot}, apparent V_d, $t_{1/2}$ and k_e values between the three doses. The $t_{1/2}$ values were 1.05, 1.1 and 1.03 hr (i.e., 63, 66 and 61 minutes, respectively) after increasing s.c. administration of rHV2. The MRT values were 3.8, 3.28 and 3.01 hrs., after administration of 0.25, 0.5 and 1.0 mg/kg, respectively. The Cl_{tot} and apparent V_d parameters were 167, 156 and 156 mL/hr/kg, and 228, 248 and 233 mL/kg, after escalating s.c. doses of rHV2. The average F value after s.c. administration of three doses of rHV2 was almost equal to 98%.

The results from coagulation tests after s.c. administration are depicted

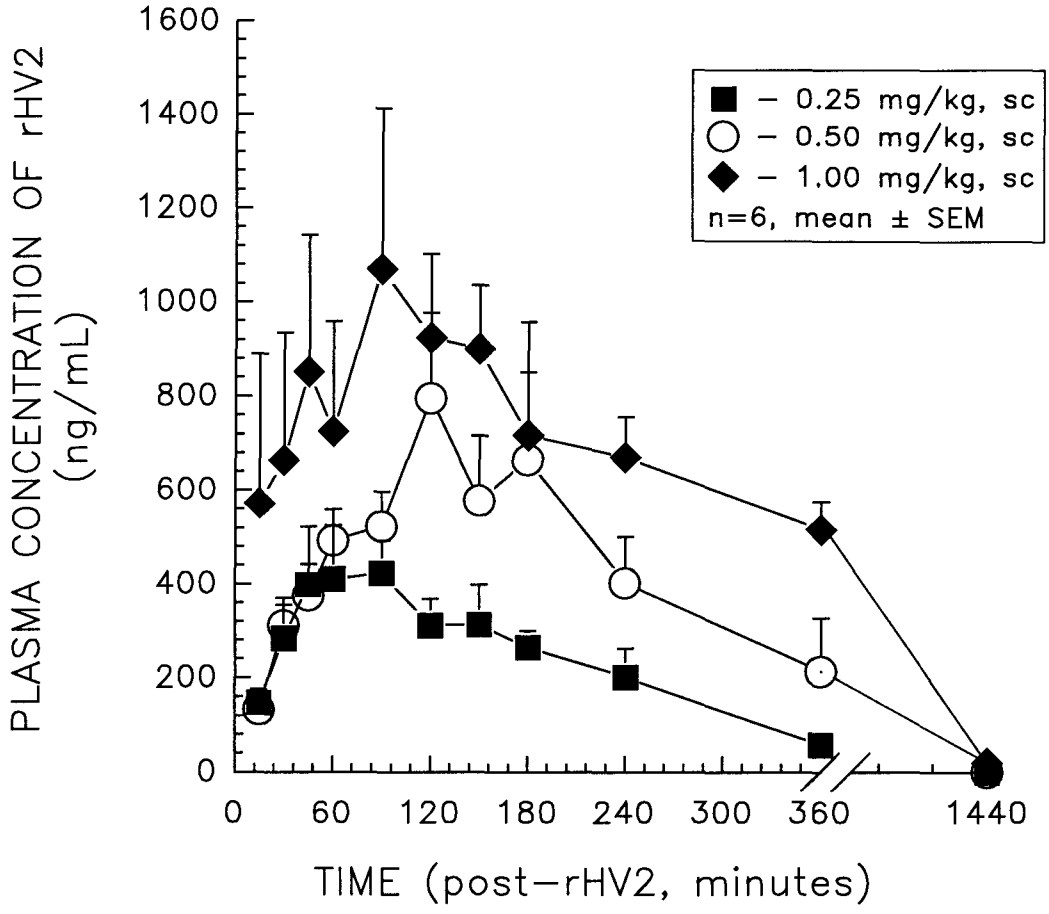


Figure 61. Pharmacokinetics of rHV2 in dogs after single s.c. bolus administration. Plasma concentrations were determined using competitive ELISA method. rHV2 was administered in s.c. doses of 0.25 (■), 0.5 (○) and 1.0 (◆) mg/kg. All data represent the mean concentration from 6 dogs ± SEM, each determined in duplicate.

TABLE 18

PHARMACOKINETIC PARAMETERS OBTAINED AFTER SINGLE DOSE S.C. (0.25, 0.50 and 1.0 mg/kg) ADMINISTRATION OF rHV2 IN DOGS

Parameter	0.25 mg/kg	0.50 mg/kg	1.0 mg/kg
C_{max} (μg/mL)	0.43	0.80	1.07
t_{max} (min)	90.00	120.00	90.00
k_e (hr^{-1})	0.73	0.63	0.67
$t_{1/2}$ (hr)	1.05	1.10	1.03
$AUC_{0-\infty}$ (μg-hr/mL)	1.75	3.04	5.95
$AUMC_{0-\infty}$ (μg-hr^2/mL)	6.67	9.97	17.90
MRT (hr)	3.80	3.28	3.01
Cl_{tot} (mL/hr/kg)	167.00	156.19	156.00
V_d (mL/kg)	228.00	247.93	232.59

in Figures 62 to 66. In the case of APTT (OT), there was a 16 to 20% increase from baseline at 75 and 180 minutes in the treatment group with the lowest dose of rHV2 (Figure 62). The baseline clotting time using APTT (OT) was about 16 seconds. The time course of APTT (OT) after the higher two doses was almost superimposable with a peak response of about 38% at 75 minutes.

Figure 63 shows the time course of APTT (Dade) after rHV2 administration. The baseline APTT (Dade) was about 12 seconds. A peak increase in activity (15% increase from baseline) was seen at 120 minutes after administration of the lowest dose. As seen with APTT (OT), the peak responses after the higher two doses were almost superimposable (22% increase at 75 minutes). After the peak response, APTT (Dade) values decreased dose dependently in these two groups (0.5 and 1 mg/kg doses).

The time course of TT (20 U/mL) is depicted in Figure 64. The baseline TT (20 U/mL) was about 7.5 seconds. Figure 64 shows that there was a dose dependent increase in TT (20 U/mL) with increasing doses of rHV2. There was almost no change in response with time with the lowest dose. With the administration of 0.5 mg/kg, there was plateau in the maximal response between 60 and 180 minutes ($\geq$ 300 seconds in clotting time). The peak response with 1.0 mg/kg was almost double as that obtained with 0.5 mg/kg. The time course of TT (20 U/mL) after 1 mg/kg also exhibited a plateau phase between 120 and 180 minutes.

The time dependent progression of results from *ex vivo* coagulation tests using Ca^{+2}TT (10 U/mL) and Ca^{+2}TT (20 U/mL) are shown in Figures 65 and 66, respectively. The baseline Ca^{+2}TT (10 U/mL) was about 10 seconds. After rHV2 administration, there was a maximal increase in Ca^{+2}TT (10 U/mL) at 120, 150 and 120 minutes using doses of 0.25, 0.5 and 1.0 mg/kg, respectively. The corresponding responses were about 8-, 28-, and 45-fold increases from baseline Ca^{+2}TT (10 U/mL), respectively (Figure 65). The baseline Ca^{+2}TT (20 U/mL) was about 6 seconds. Figure 66 shows that there was almost no difference in Ca^{+2}TT (20 U/mL) from baseline at all blood sampling times after administration of the lowest dose. Maximal Ca^{+2}TT (20 U/mL) responses were obtained at 75 and 120 minutes after rHV2 administration of 0.5 and 1.0 mg/kg, respectively.

The variations in *ex vivo* antithrombin activity (anti-IIa assay) with time are depicted in Figure 67. There was a linear increase in thrombin inhibitory response with increasing doses of rHV2. However, the maximal responses obtained with the s.c. doses were lesser than that obtained with corresponding i.v. doses of rHV2 (see Figure 60). The peak thrombin inhibitory activities were about 18% (between 75 and 150 minutes), 34% (at 75 minutes) and 50% (at 120 minutes) after rHV2 administration of 0.25, 0.5 and 1.0 mg/kg, respectively.

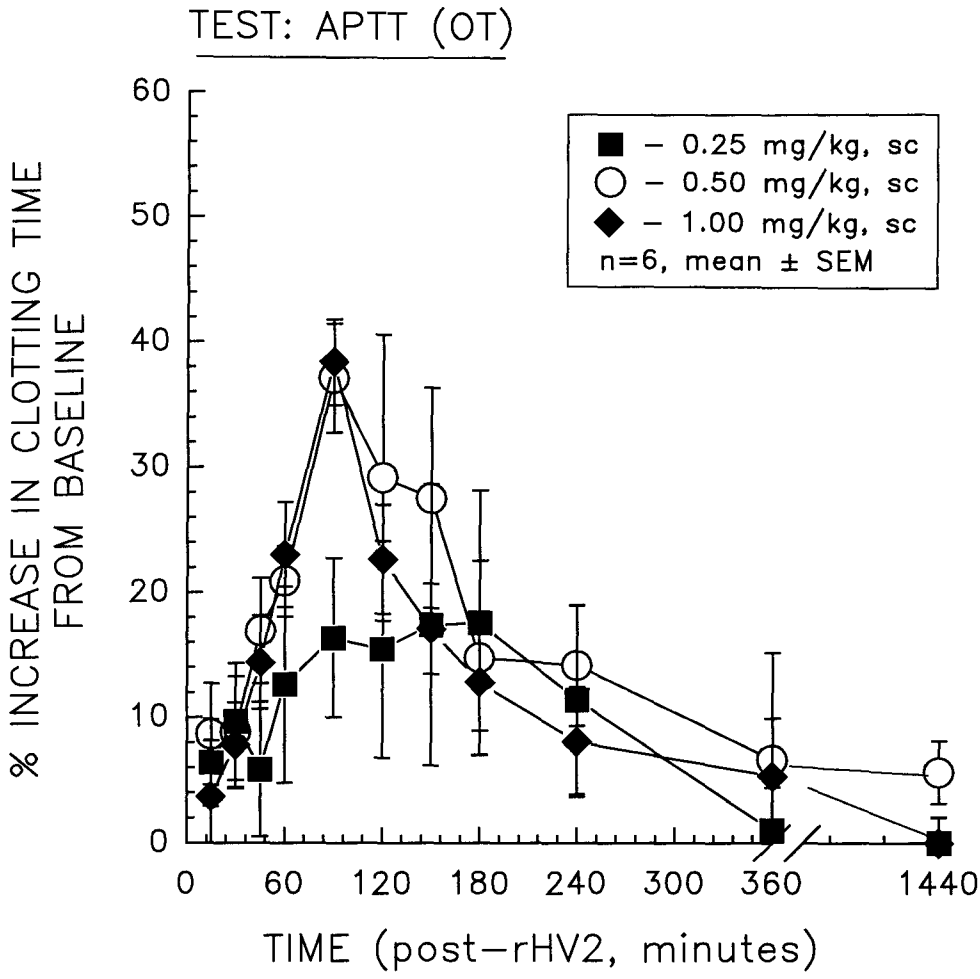


Figure 62. Time-course of *ex vivo* antithrombin activity [APTT (OT)] of rHV2 after administration increasing s.c doses in dogs. rHV2 was used in doses of 0.25 (■), 0.5 (○) and 1.0 (◆) mg/kg. All data represent the mean concentration from 6 dogs ± SEM, each determined in duplicate.

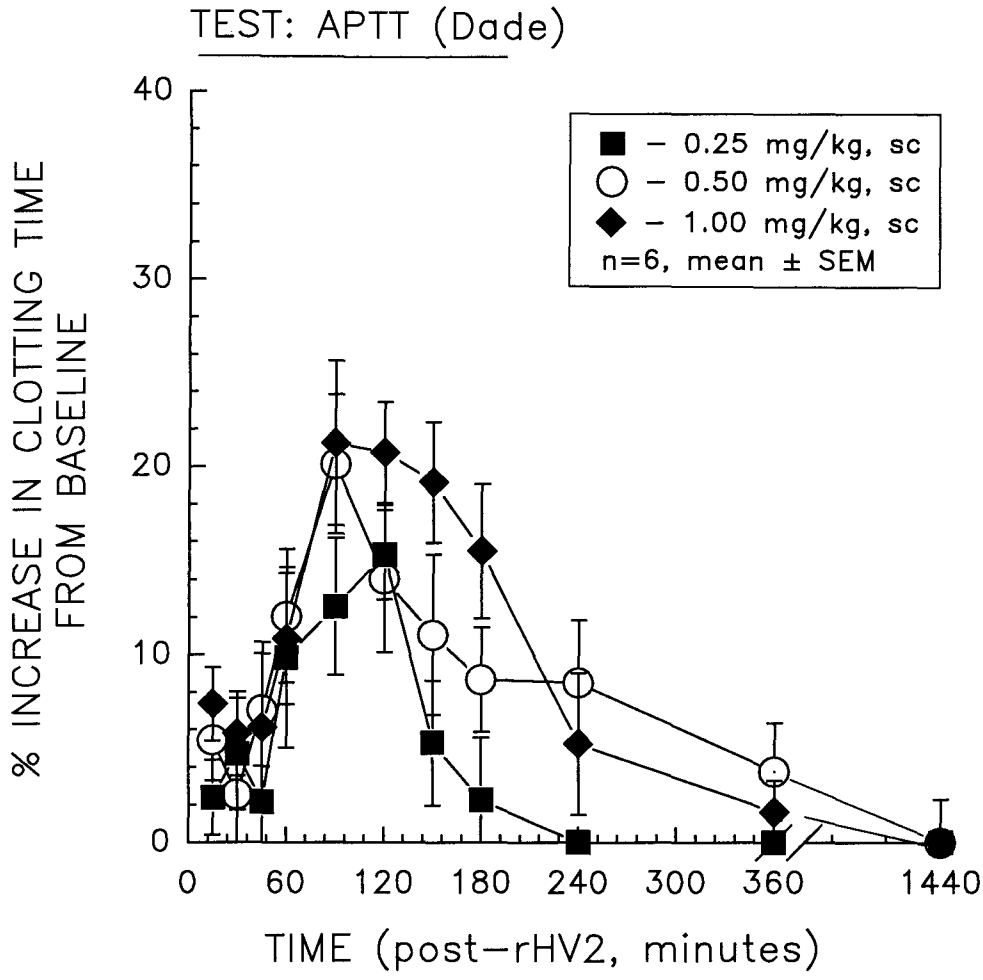


Figure 63. Time-course of *ex vivo* antithrombin activity [APTT (Dade)] of rHV2 after administration increasing s.c. doses in dogs. rHV2 was used in doses of 0.25 (■), 0.5 (○) and 1.0 (◆) mg/kg. All data represent the mean concentration from 6 dogs ± SEM, each determined in duplicate.

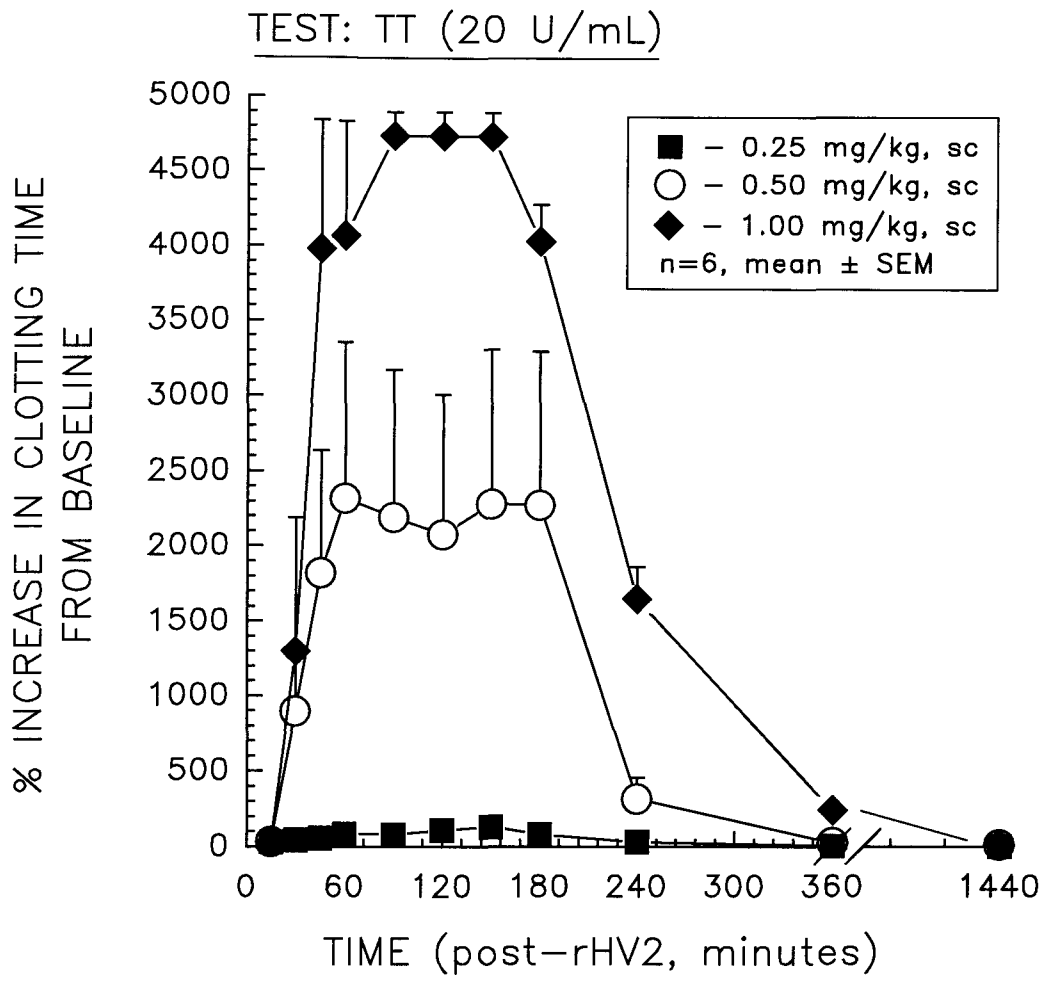


Figure 64. Time-course of *ex vivo* antithrombin activity TT (20 U/mL) of rHV2 after administration increasing s.c. doses in dogs. rHV2 was used in doses of 0.25 (■), 0.5 (○) and 1.0 (◆) mg/kg. All data represent the mean concentration from 6 dogs ± SEM, each determined in duplicate.

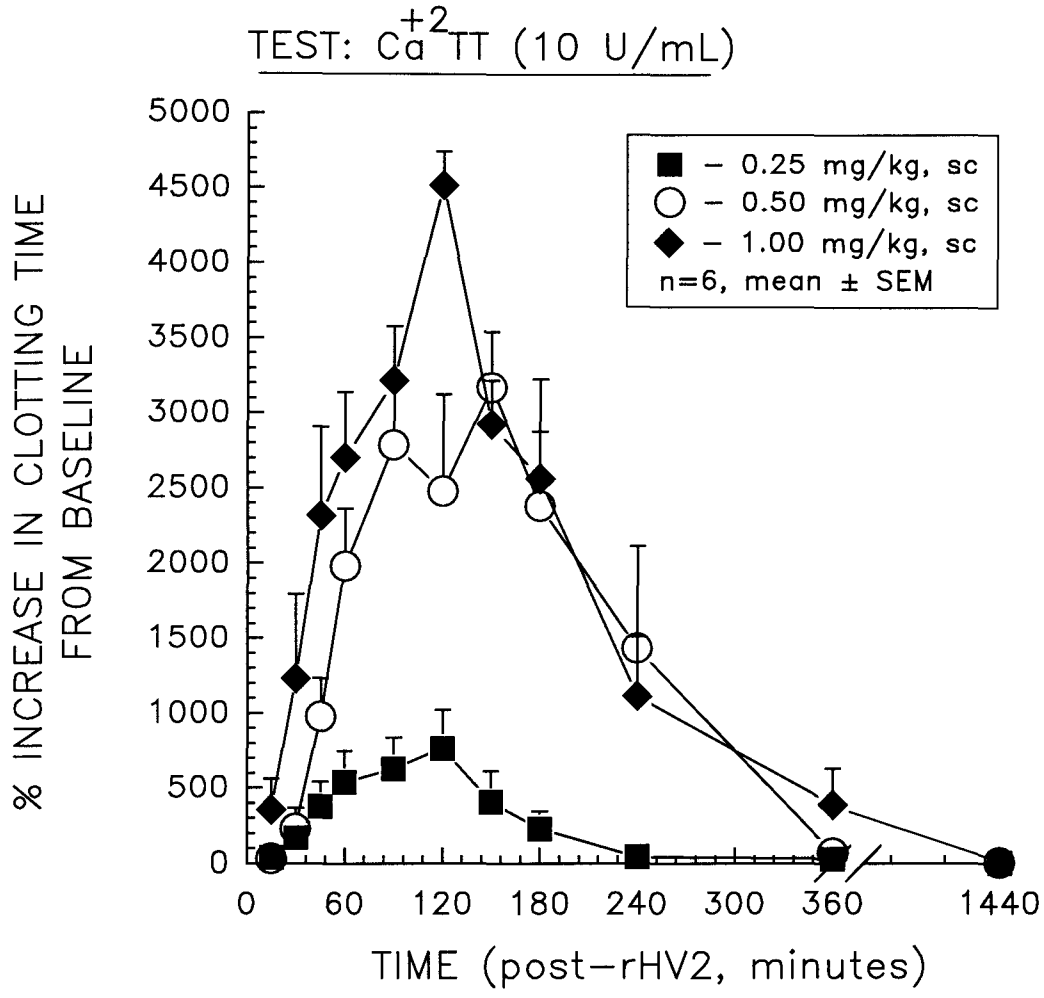


Figure 65. Time-course of *ex vivo* antithrombin activity $Ca^{+2}TT$ (10 U/mL) of rHV2 after administration increasing s.c. doses in dogs. rHV2 was used in doses of 0.25 (■), 0.5 (○) and 1.0 (◆) mg/kg. All data represent the mean concentration from 6 dogs ± SEM, each determined in duplicate.

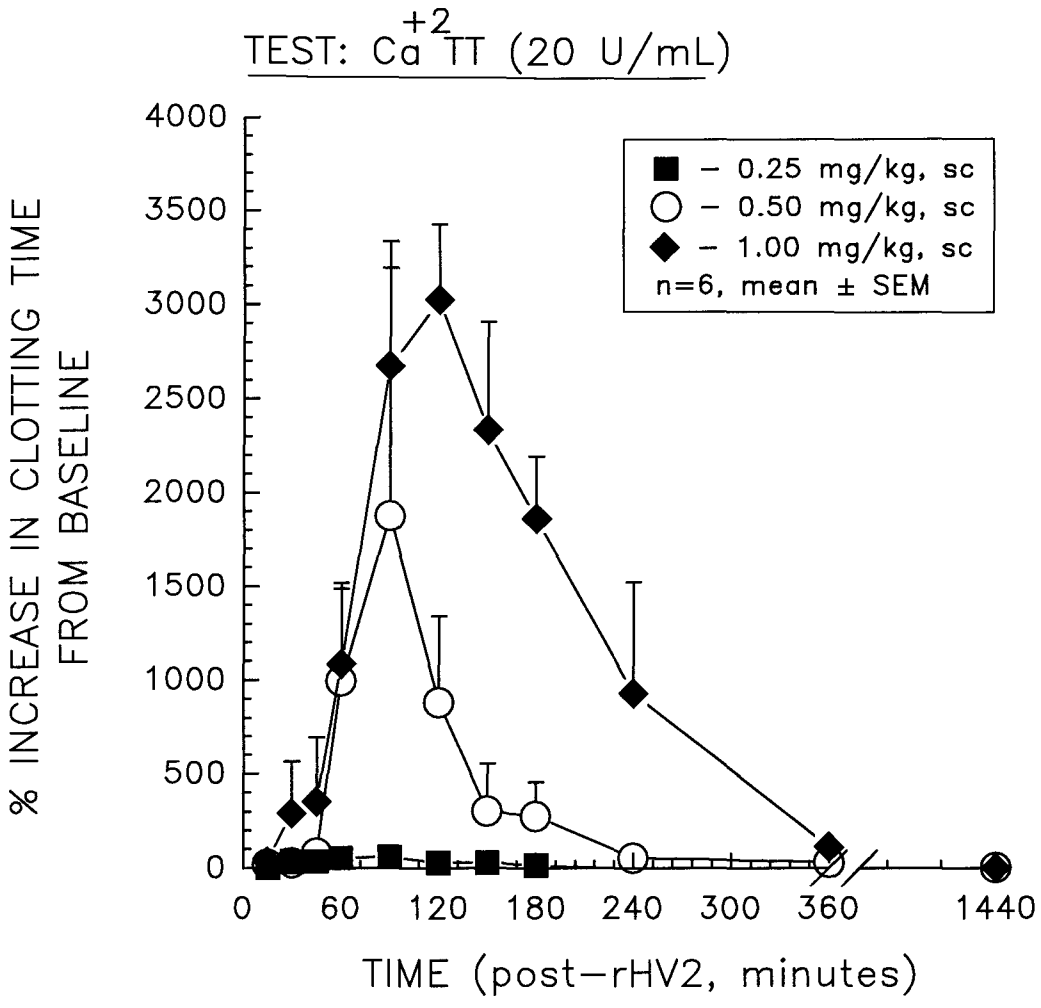


Figure 66. Time-course of *ex vivo* antithrombin activity $Ca^{+2}TT$ (20 U/mL) of rHV2 after administration increasing s.c. doses in dogs. rHV2 was used in doses of 0.25 (■), 0.5 (○) and 1.0 (◆) mg/kg. All data represent the mean concentration from 6 dogs ± SEM, each determined in duplicate.

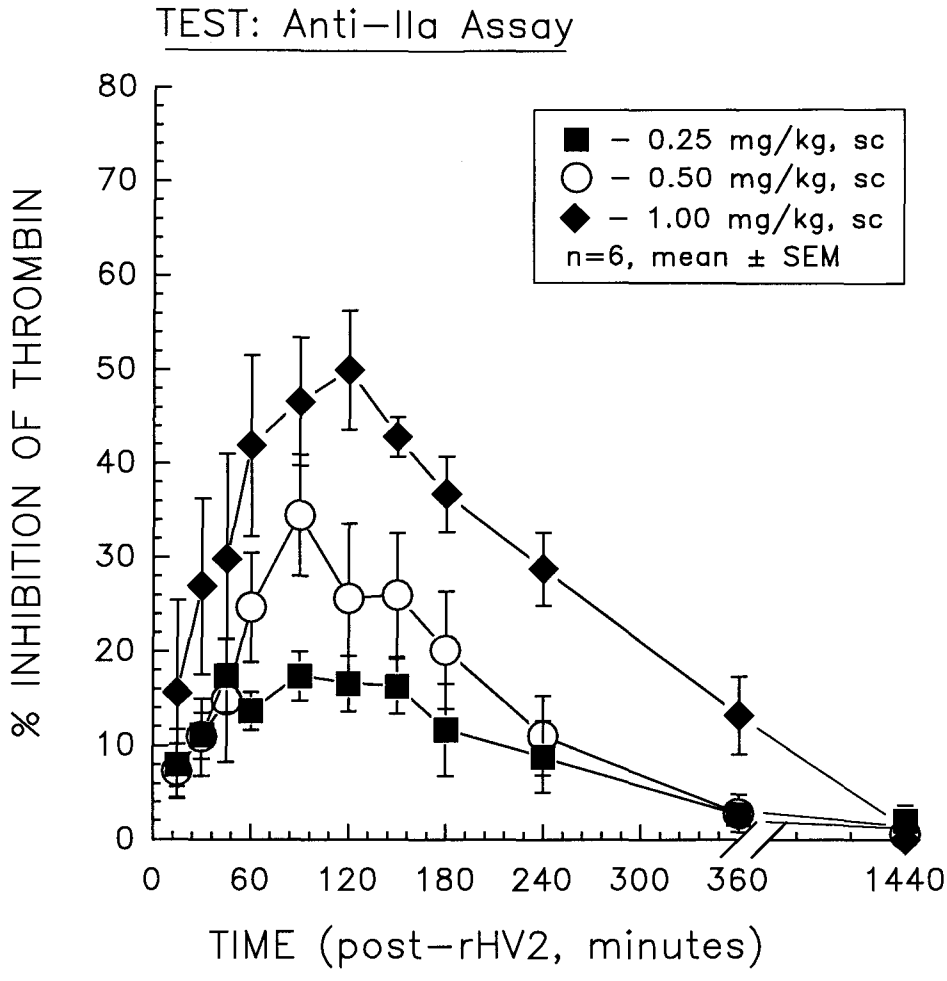


Figure 67. Time-course of *ex vivo* antithrombin activity of rHV2 after administration increasing s.c. doses in dogs (chromogenic anti-IIa assay). rHV2 was used in doses of 0.25 (■), 0.5 (○) and 1.0 (◆) mg/kg. Antithrombin activity was determined using chromogenic anti-IIa assay. All data represent the mean concentration from 6 dogs ± SEM, each determined in duplicate.

7. Investigation of Pharmacodynamics of rHV2 after Repeated Administration in Dogs

rHV2 was administered at a repeated, daily dose of 1 mg/kg for a period of seven days using i.v. and s.c. routes (n = 6 dogs, per route). Plasma samples obtained after specified time intervals were subjected to the competitive ELISA, chromogenic anti-IIa assay and coagulation tests, as described in the chapter on "Materials, Methods and Experimental Protocols".

I.V. Administration. The time course of plasma concentration of rHV2 after multiple dose administration in dogs, is depicted in Figure 68. The symbols: d, a, b, and c, indicate the plasma level of rHV2 at baseline, 6, 30 and 60 minutes after administration of each dose. After administration of the first dose (I), approximately 7.4 μg/mL of rHV2 was detected in the plasma by the ELISA method at 6 minutes. As observed with the single dose i.v. experiments, there was a rapid decline in the plasma concentrations after 6 minutes, decreasing to approximately 2 and 1 μg/mL at 30 and 60 minutes, respectively. After administration of doses II and III, the plasma rHV2 levels at 6 minutes increased to $\approx$ 11 and 10.8 μg/mL, respectively. However, there was no significant difference between the rHV2 levels between all seven doses at 6 minutes after drug administration ($p = 0.301$, one-way repeated measures ANOVA). The plasma concentrations of rHV2 at 30 and 60 minutes after each dose were also not significantly different from each other (one-way repeated measures ANOVA).

The area under the plasma concentration-time curve and clearance values were calculated from baseline to 24 hours after each dose. These values are listed in Table 19.

TABLE 19

AREA UNDER THE CURVE AND CLEARANCE VALUES AFTER REPEATED ADMINISTRATION (1 mg/kg) OF rHV2 IN DOGS

Dose	I.V. Dosing		S.C. Dosing	
	AUC (μg-hr/mL)	Cl_{tot} (mL/hr/kg)	AUC (μg-hr/mL)	Cl_{tot} (mL/hr/kg)
I	11.84 ± 0.50	85.08 ± 3.47	5.37* ± 1.46	120.45* ± 29.44
II	17.00 ± 3.21	66.52 ± 9.30	7.75 ± 0.93	137.78* ± 28.01
III	19.42 ± 4.95	65.75 ± 12.71	9.21 ± 0.68	49.48 ± 3.04
IV	15.16 ± 4.46	88.55 ± 19.09	8.17 ± 0.92	65.04 ± 9.2
V	17.18 ± 4.07	71.32 ± 13.34	7.44 ± 1.13	68.38 ± 11.07
VI	13.36 ± 0.46	75.26 ± 2.67	9.46 ± 1.57	51.24 ± 4.63
VII	15.44 ± 3.74	77.99 ± 13.15	9.49 ± 1.19	50.67 ± 5.35

* - $p<0.05$, one-way repeated measures ANOVA
All data represent mean observation from six dogs ± SEM

After i.v. dosing, there were no significant differences in the AUC as well as clearance values with repeated administration ($p = 0.597$/AUC and 0.530/Cl_{tot}, one-way repeated measures ANOVA).

A similar progression in *ex vivo* antithrombin activity was obtained with the APTT (Dade). The results with APTT (Dade) are illustrated in Figure 69. There was a sharp increase in APTT (Dade) at 6 minutes after each dose of rHV2, followed by a rapid decrease in activity at 30 minutes and 60 minutes. The step-wise decrease observed in the progression of plasma rHV2 concentrations and APTT (Dade) was not as clearly evident with the Ca^{+2}TT (10 U/mL) assay. The time course of Ca^{+2}TT (10 U/mL) after repeated rHV2 administration is shown in Figure 70. At each blood sampling time, there was no significant difference in APTT (Dade) as well as Ca^{+2}TT (10 U/mL) between all seven doses (one-way repeated measures ANOVA).

Figure 71 illustrates the time course of *ex vivo* antithrombin activity of rHV2 after repeated i.v. administration in dogs, using the amidolytic anti-IIa assay. An 82% inhibition of thrombin was obtained at 6 minutes after administration of dose I. After administration of the subsequent doses, the thrombin inhibitory activity increased to approximately 96-98%. No significant differences were found in the thrombin inhibitory activity at 6, 30 and 60 minutes after each dose (one-way repeated measures ANOVA).

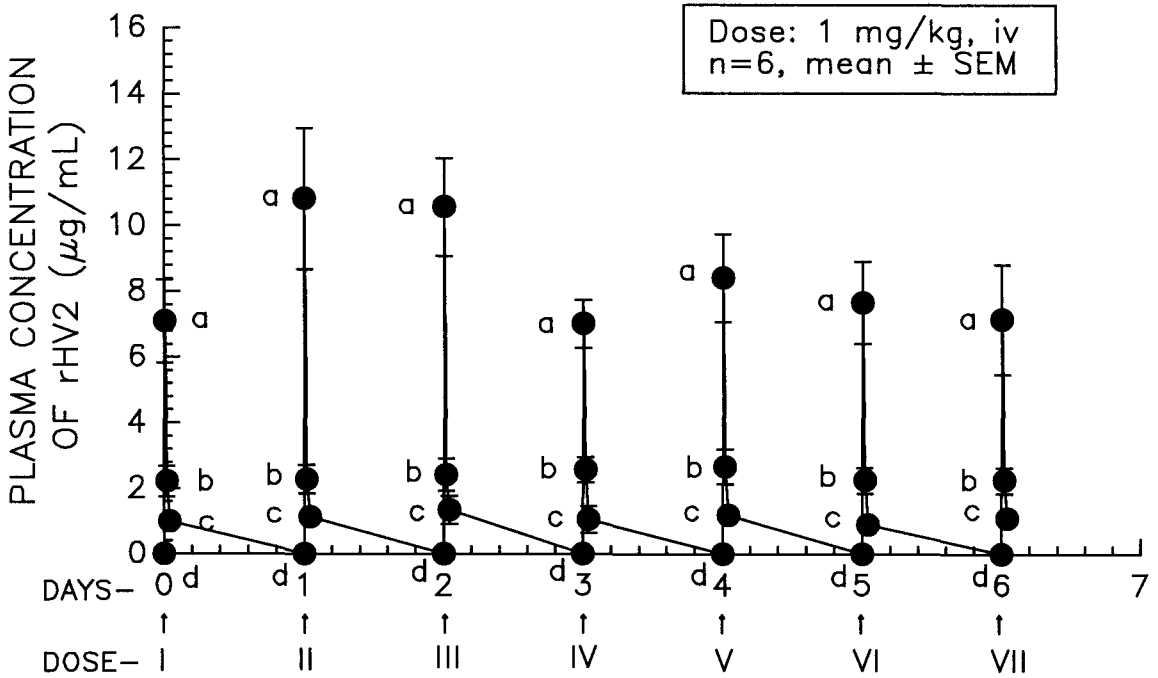


Figure 68. Time-course of plasma concentration of rHV2 after repeated i.v. administration in dogs. rHV2 was administered in daily doses of 1 mg/kg for one week. Plasma concentrations were determined using the competitive ELISA method. Each observation represents the mean concentration from six dogs ± SEM.

d, a, b, and c - baseline, 6, 30, 60 minutes after each dose, respectively.

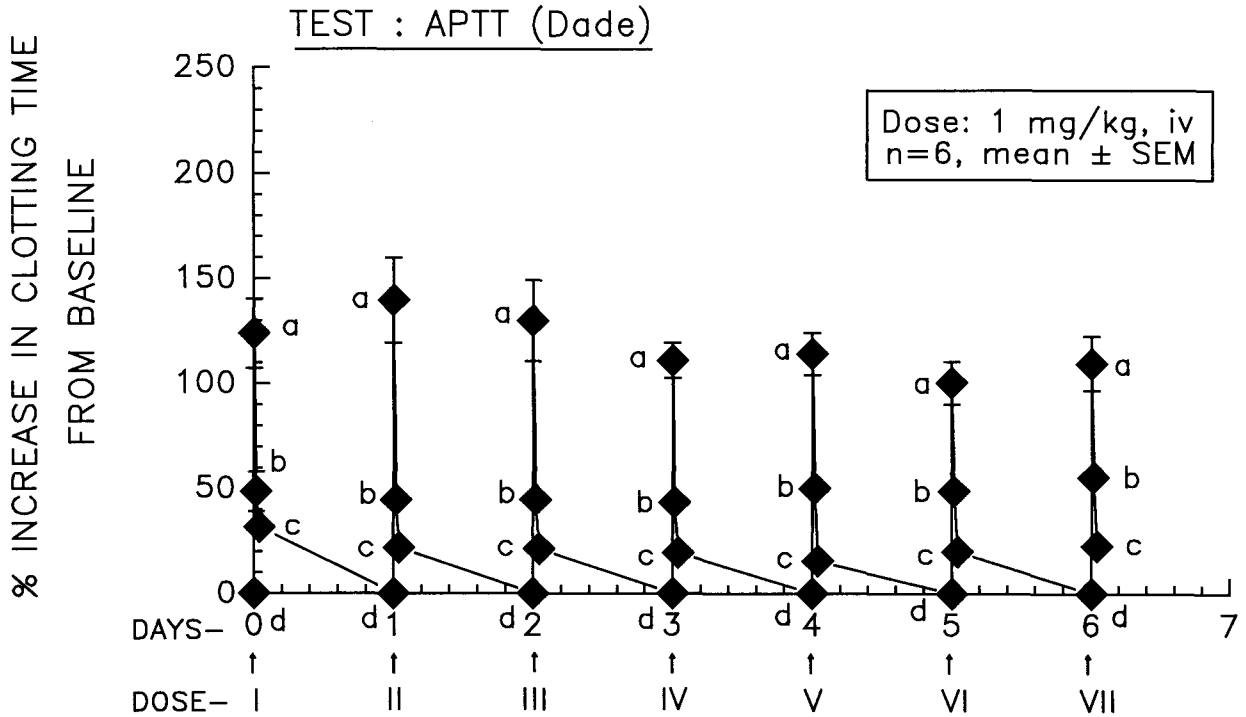


Figure 69. Time-course of *ex vivo* antithrombin activity (APTT-Dade) of rHV2 after repeated i.v. administration in dogs. rHV2 was administered in daily doses of 1 mg/kg for one week. Each observation represents the mean observation from six dogs ± SEM, each determined in duplicate.

d, a, b, and c - baseline, 6, 30, 60 minutes after each dose, respectively.

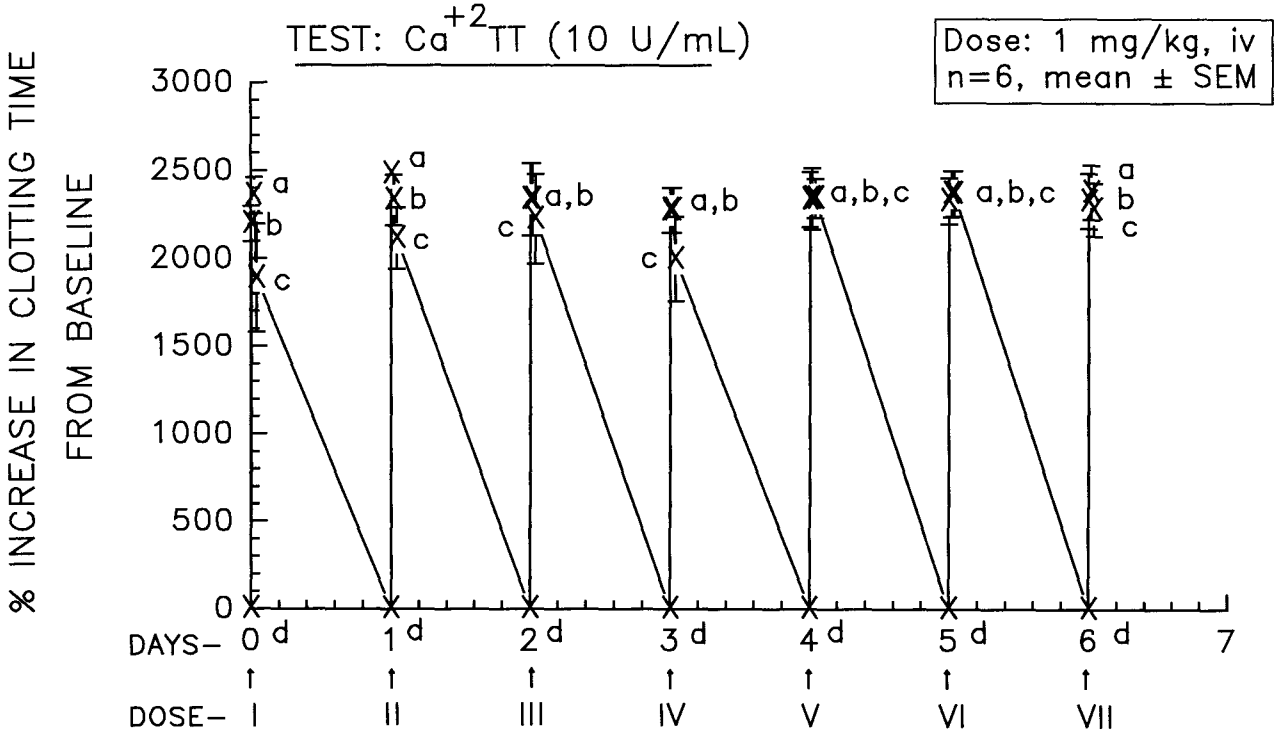


Figure 70. Time-course of *ex vivo* antithrombin activity (Ca^{+2}TT-10 U/mL) of rHV2 after repeated i.v. administration in dogs. rHV2 was administered in daily doses of 1 mg/kg for one week. Each observation represents the mean observation from six dogs ± SEM, each determined in duplicate.

d, a, b, and c - baseline, 6, 30, 60 minutes after each dose, respectively.

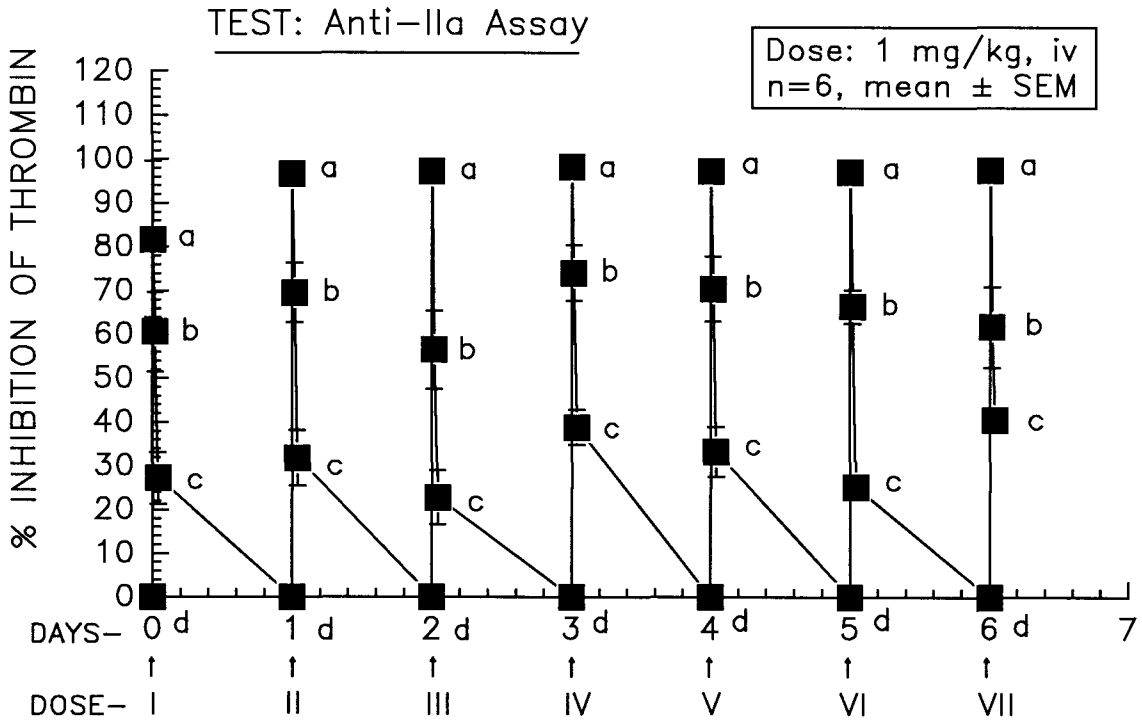


Figure 71. Time-course of *ex vivo* antithrombin activity (chromogenic anti-IIa assay) of rHV2 after repeated i.v. administration in dogs. rHV2 was administered in daily doses of 1 mg/kg for one week. Each observation represents the mean observation from six dogs ± SEM, each determined in duplicate.
d, a, b, and c - baseline, 6, 30, 60 minutes after each dose, respectively.

S.C. Administration. Blood samples were drawn at baseline and at 120, 180 and 240 minutes after each s.c. dose of rHV2. Figure 72 describes the time course of plasma concentrations of rHV2 after repeated s.c. administration. The symbols: s, p, q, and r, represent plasma levels of rHV2 at baseline, 120, 180, and 240 minutes after each dose. A comparison of rHV2 levels at 240 minutes indicated no difference between the seven doses ($p = 0.239$, one-way repeated measures ANOVA). There were no significant differences in measured plasma concentrations of rHV2 between the seven, repeated administrations at 120 and 180 minutes, as well (one-way repeated measures ANOVA).

The AUC and Cl_{tot} values were calculated after each s.c. dose, the values of which are listed in Table 19. There was a significant increase in AUC values after the second dose ($p = 0.023$, one-way repeated measures ANOVA). In addition, there was a significant decrease in Cl_{tot} after the third dose ($p = 0.000$, one-way repeated measures ANOVA).

The progression of APTT (Dade) and Ca^{+2}TT (10 U/mL) after repeated s.c. administration of rHV2 is shown in Figures 73 and 74, respectively. Using both tests, there was no significant variation in the *ex vivo* antithrombin activity at each blood sampling time, after each dose of rHV2 (one-way repeated measures ANOVA).

The anti-IIa assay exhibited a more step-wise progression of thrombin inhibitory activity than the APTT (Dade) and Ca^{+2}TT (10 U/mL) assay (Figure

75). Similar to previous results, no significant differences were observed in the responses between doses, at various blood sampling times (one-way repeated measures ANOVA). As seen in the single dose s.c. studies, only a 50 to 55% maximum inhibition of thrombin was observed at 120 minutes.

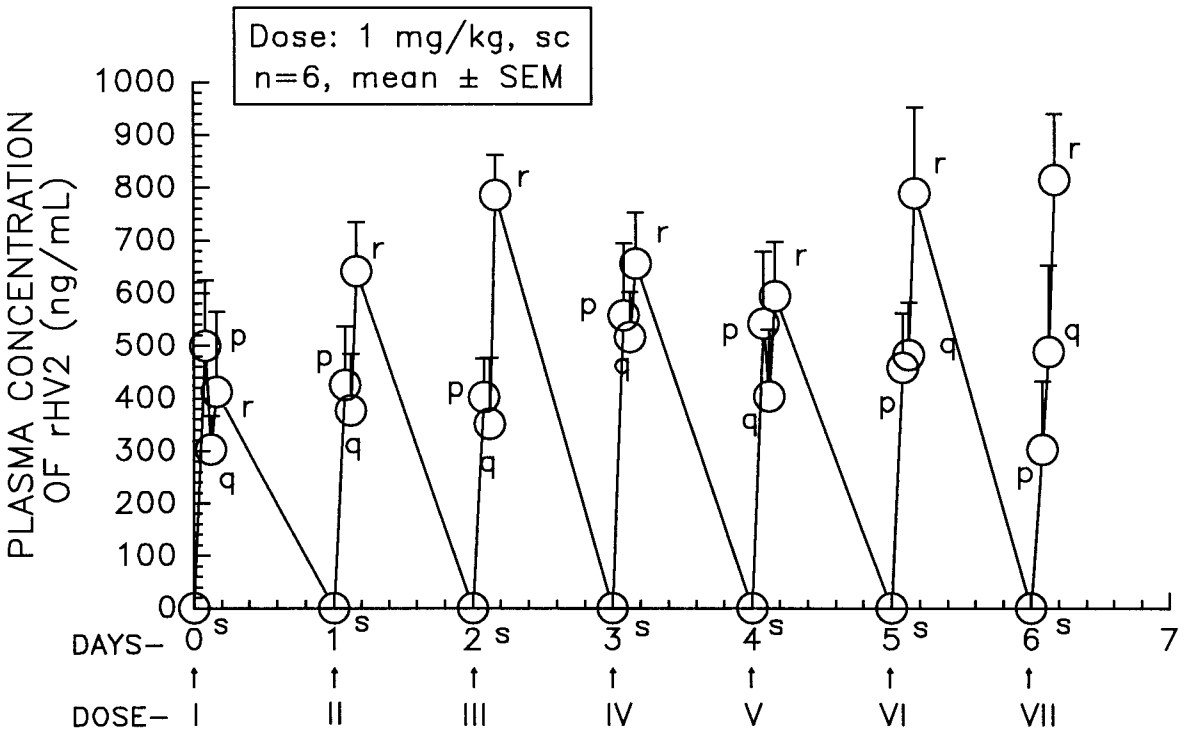


Figure 72. Time-course of plasma concentration of rHV2 after repeated s.c. administration in dogs. rHV2 was administered in daily doses of 1 mg/kg for one week. Plasma concentrations were determined using the competitive ELISA method. Each observation represents the mean concentration from six dogs ± SEM.

s, p, q, and r - baseline, 120, 180 and 240 minutes, after each dose, respectively.

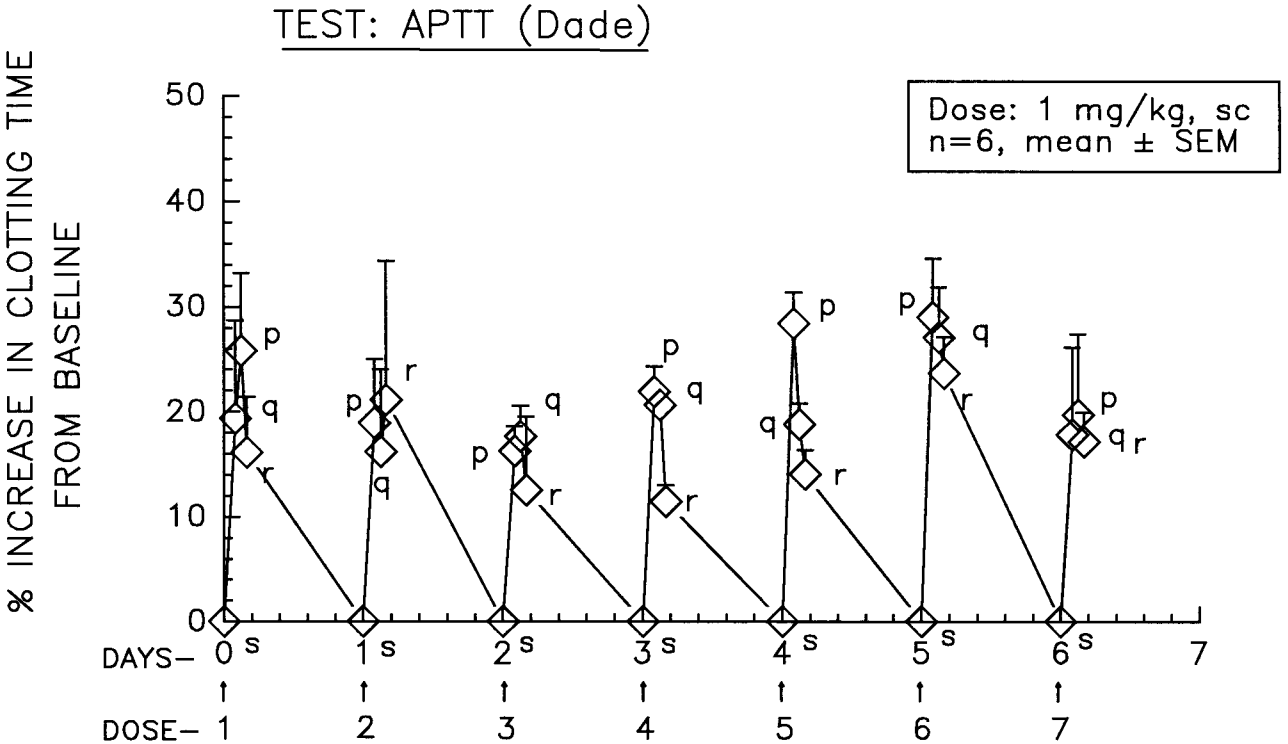


Figure 73. Time-course of *ex vivo* antithrombin activity (APTT-Dade) of rHV2 after repeated s.c. administration in dogs. rHV2 was administered in daily doses of 1 mg/kg for one week. Each observation represents the mean observation from six dogs ± SEM, each determined in duplicate.

s, p, q, and r - baseline, 120, 180 and 240 minutes, after each dose, respectively.

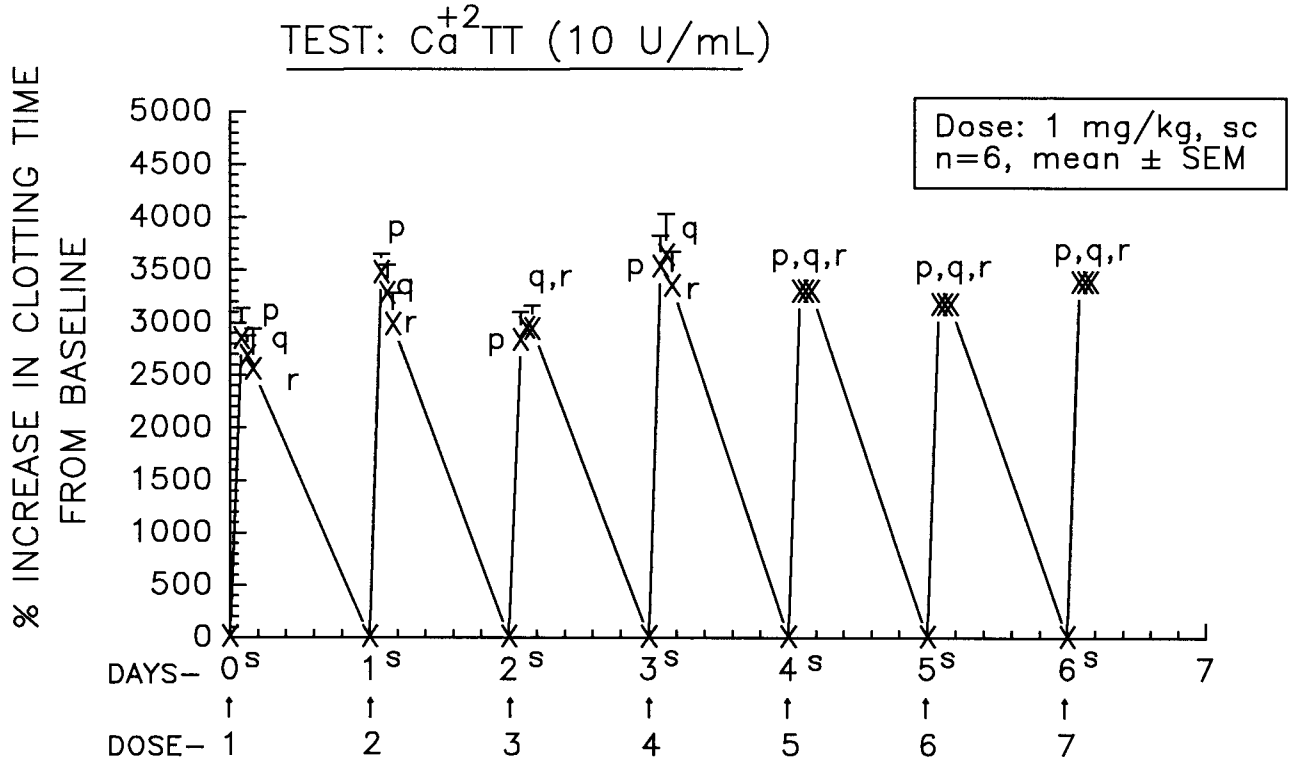


Figure 74. Time-course of *ex vivo* antithrombin activity (Ca^{+2}TT-10 U/mL) of rHV2 after repeated s.c. administration in dogs. rHV2 was administered in daily doses of 1 mg/kg for one week. Each observation represents the mean observation from six dogs ± SEM, each determined in duplicate.

baseline, p, q, and r - 120, 180 and 240 minutes, after each dose, respectively.

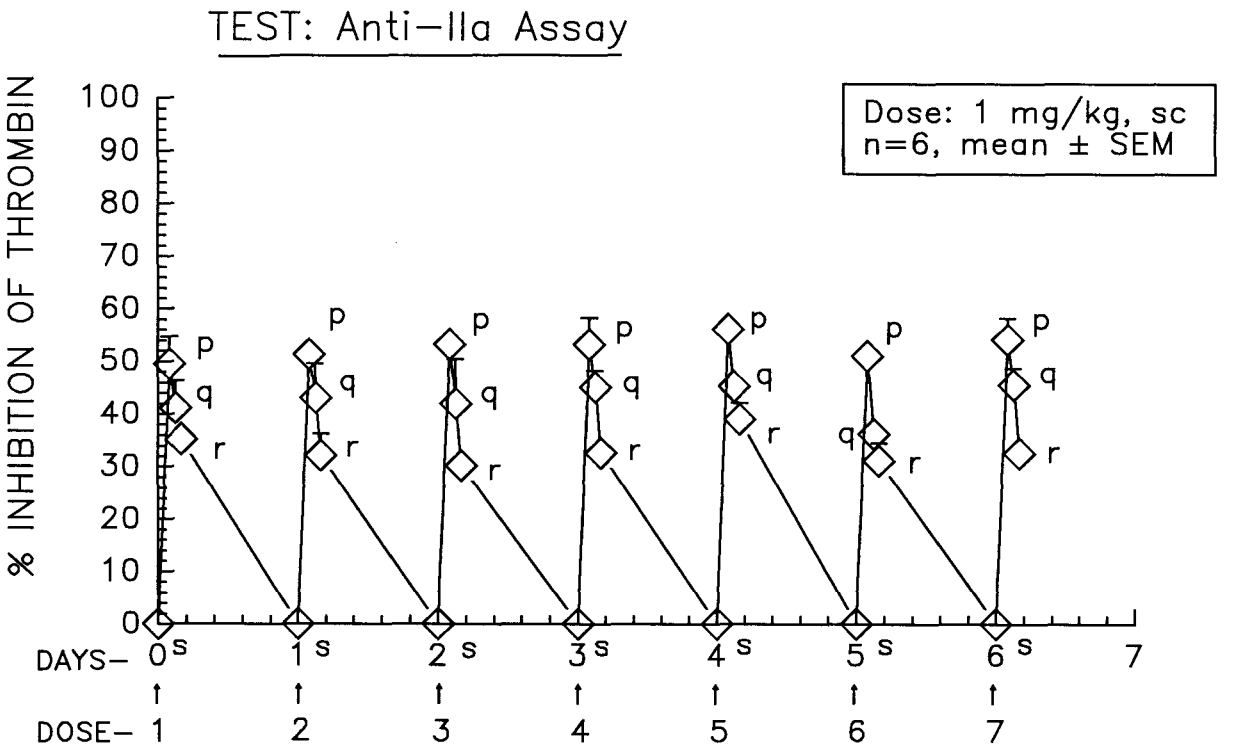


Figure 75. Time-course of *ex vivo* antithrombin activity (chromogenic anti-IIa assay) of rHV2 after repeated s.c. administration in dogs. rHV2 was administered in daily doses of 1 mg/kg for one week. Each observation represents the mean observation from six dogs ± SEM, each determined in duplicate. baseline, p, q, and r - 120, 180 and 240 minutes, after each dose, respectively.

7a. Serum Chemistry Profile in Dogs after Repeated rHV2 Administration

I.V. Administration. Male, mongrel dogs were treated with repeated i.v. administration of rHV2 (1 mg/kg, every 24 hours) for seven days, as described under "Materials, Methods and Experimental Protocols". Figures 76 to 80 illustrate the variations in different serum chemistry parameters at baseline ("pre-rHV2") and 30 minutes ("post-rHV2") after rHV2 administration over this period. For each parameter, a two-way repeated analysis of variance was performed, to study the effect of day (rHV2 dose on each day) of treatment ("day effect"), the difference between "pre-rHV2" and "post-rHV2" on each day ("pre-post effect") as well as that of any interaction between the two effects.

Figure 76 depicts changes in phosphorous, albumin, uric acid and total bilirubin levels at baseline and 30 minutes after each dose of rHV2. No significant differences were found in any of the above parameters between the various days, and between baseline and 30 minutes after rHV2 administration, on each day. Figure 77 shows the variations in electrolytes after repeated rHV2 administration in dogs. No significant differences were found in sodium, chloride, calcium and potassium levels between different days, and pre- and post-rHV2 times of blood draw on each day.

Figure 78 illustrates the changes in hepatic enzymes, alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) as well as blood urea nitrogen (BUN) and creatinine

after repeated rHV2 administration. Once again, no significant variations were observed in these parameters, using the two-way ANOVA test.

The variations in total CO_2, total protein and γ-glutamyl transferase (γ-GT) after repeated rHV2 administration are shown in Figure 79. No significant difference was observed in total protein values between the seven days, and pre- and post-rHV2 times. However, there was a significant day-to-day variation in total CO_2 levels at the 90% confidence level ($p = 0.076$). There was a trend toward an initial increase in total CO_2 levels at pre- and post-drug times on the first two days, followed by a gradual decrease on the remaining days. Within each day, there was no significant difference between pre- and post-rHV2 total CO_2 values. Furthermore, there was no significant interaction between the day and pre-post effects on total CO_2 levels. Similarly, there was a significant day-to-day variation in γ-GT levels ($p = 0.085$) with an initial decrease in pre- and post-rHV2 levels up to day 3, followed by an increase to levels equal to initial values, by the sixth day (Figure 79).

Figure 80 depicts the variations in lactic dehydrogenase (LDH), cholesterol, glucose and triglyceride levels after repeated i.v. administration of rHV2 in dogs. As observed with most other serum chemistry parameters, there were no significant variations between all the above parameters (two-way repeated measures ANOVA). It was interesting to note that there was a gradual decrease in LDH levels up to day 3 and gradual increase thereafter to initial levels. However, this progression was not significant ($p = 0.297$,

0.520 and 0.284 for the day effect, pre-post effect and the interaction, respectively). A similar trend was observed with triglyceride levels, where a slight decrease, though insignificant, was observed on the third day of the study (Figure 80).

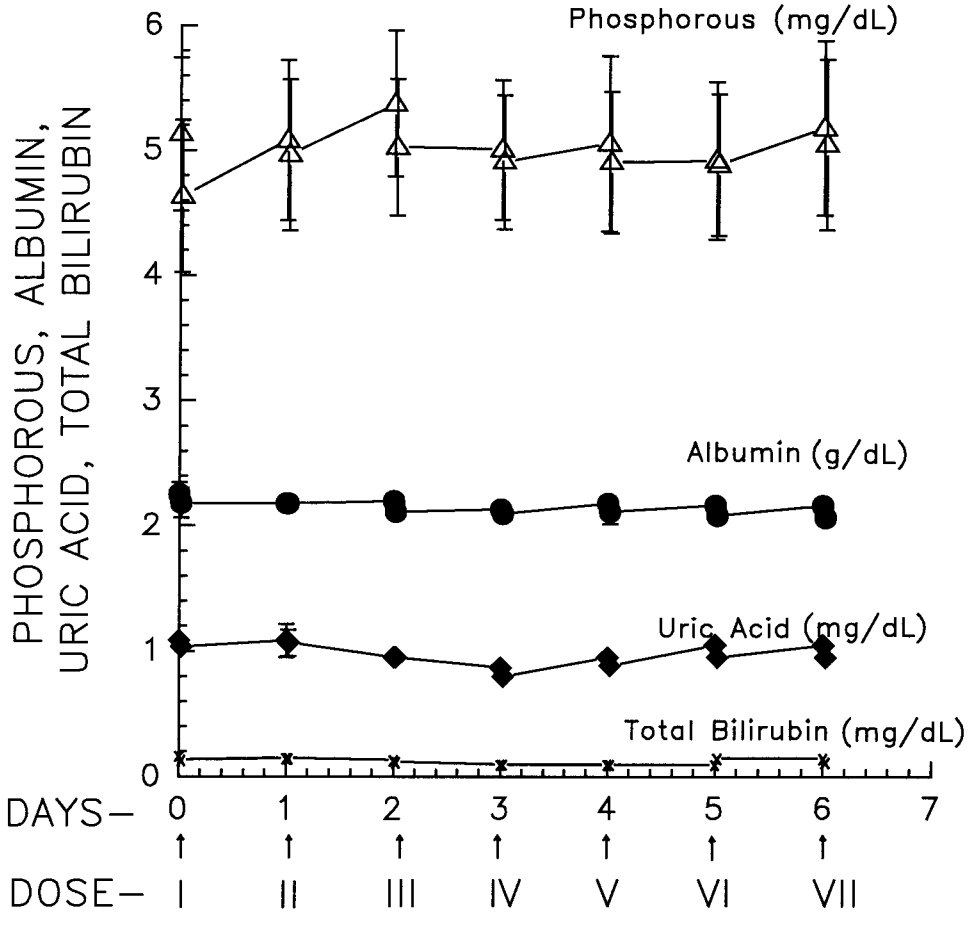


Figure 76. Serum chemistry profile after repeated i.v. administration of rHV2 in dogs (part I). Male, mongrel dogs (n=6) were injected with 1 mg/kg rHV2 every 24 hours, for a period of one week. Figure shows changes in phosphorous, albumin, uric acid and total bilirubin levels at baseline and 30 minutes after each administration. All data represent the mean determination from 6 dogs ± SEM.

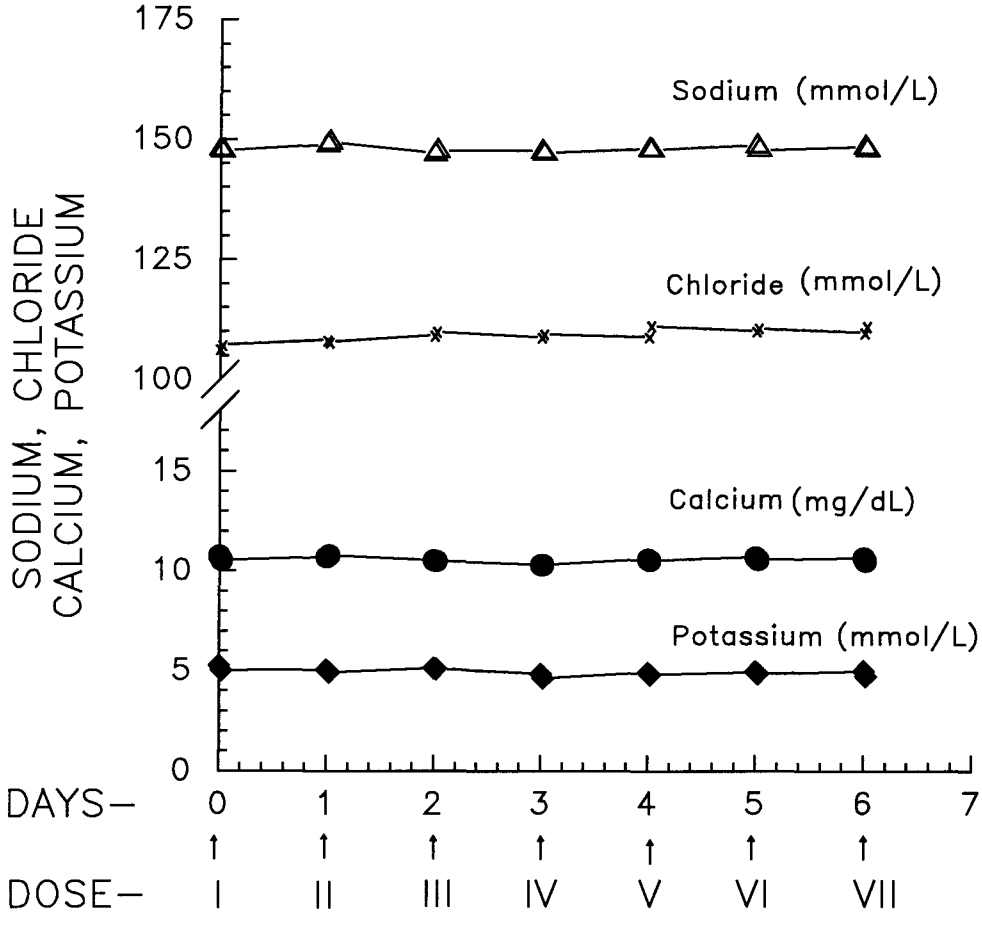


Figure 77. Serum chemistry profile after repeated i.v. administration of rHV2 in dogs (part II). Male, mongrel dogs (n = 6) were injected with 1 mg/kg rHV2 every 24 hours, for a period of one week. Figure shows changes in sodium, chloride, calcium and potassium levels at baseline and 30 minutes after each administration. All data represent the mean determination from 6 dogs ± SEM.

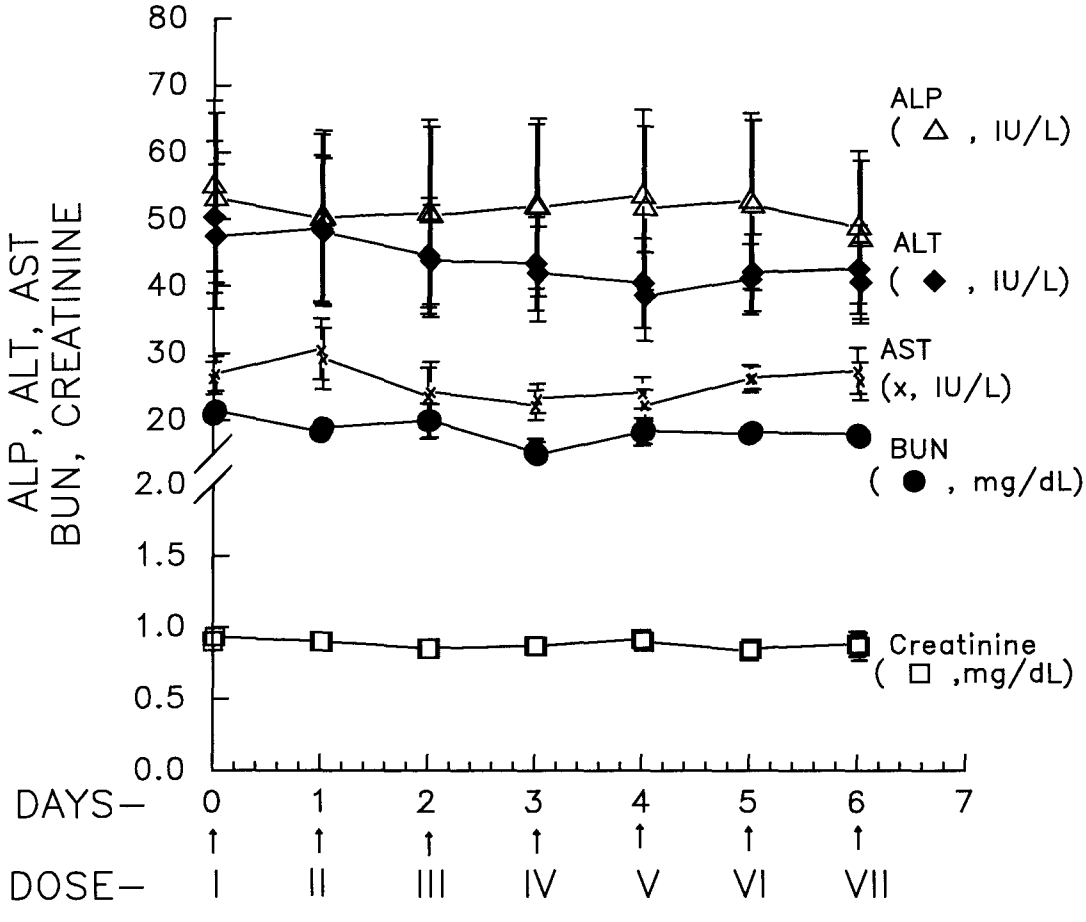


Figure 78. Serum chemistry profile after repeated i.v. administration of rHV2 in dogs (part III). Male, mongrel dogs (n = 6) were injected with 1 mg/kg rHV2 every 24 hours, for a period of one week. Figure shows changes in alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine at baseline and 30 minutes after each administration. All data represent the mean determination from 6 dogs ± SEM.

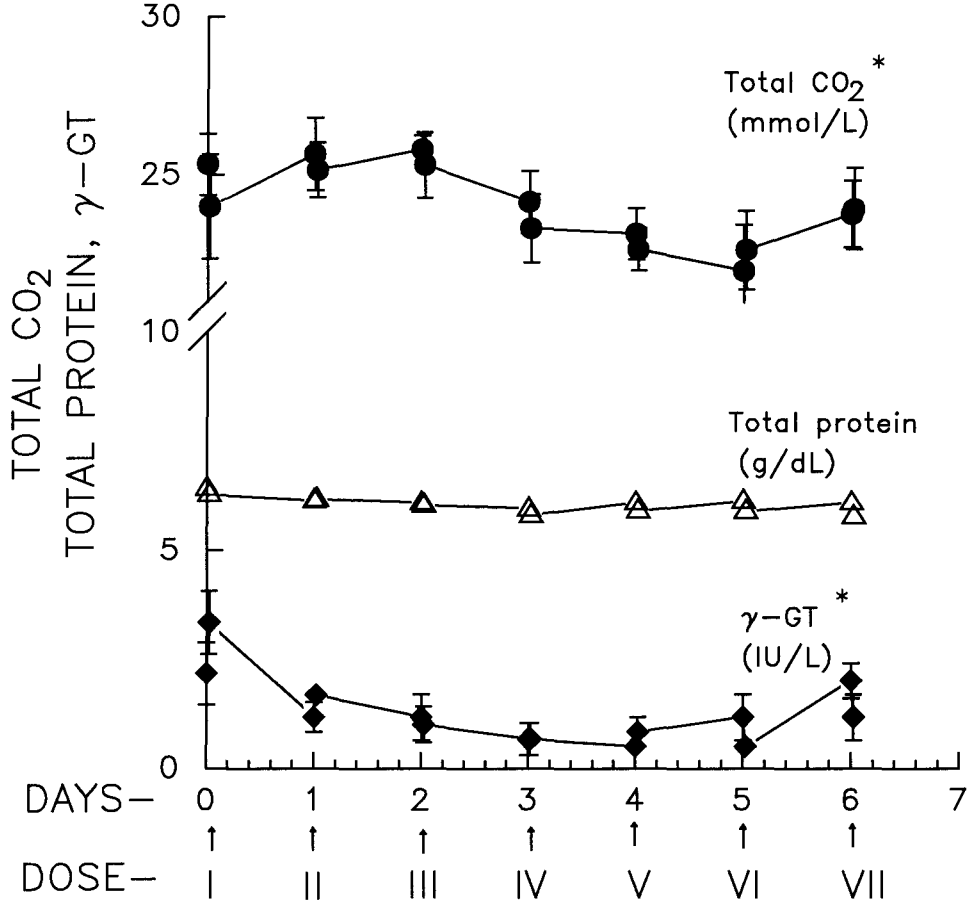


Figure 79. Serum chemistry profile after repeated i.v. administration of rHV2 in dogs (part IV). Male, mongrel dogs (n = 6) were injected with 1 mg/kg rHV2 every 24 hours, for a period of one week. Figure shows changes in total CO_2, total protein and γ-glutamyltransaminase (γ-GT) at baseline and 30 minutes after each administration. All data represent the mean determination from 6 dogs ± SEM.

*-day-to-day variation, $p < 0.10$ (two-way repeated measures ANOVA).

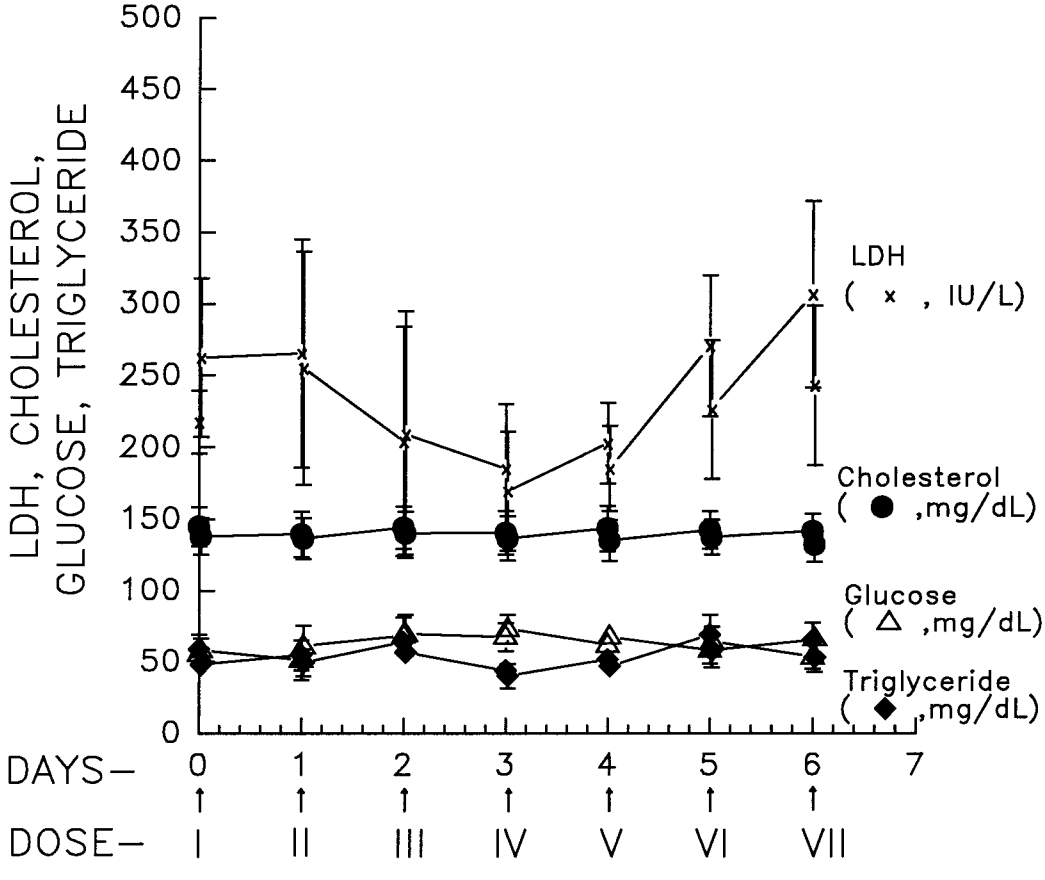


Figure 80. Serum chemistry profile after repeated i.v. administration of rHV2 in dogs (part V). Male, mongrel dogs (n = 6) were injected with 1 mg/kg rHV2 every 24 hours, for a period of one week. Figure shows changes in lactate dehydrogenase (LDH), cholesterol, glucose and triglyceride levels at baseline and 30 minutes after each administration. All data represent the mean determination from 6 dogs ± SEM.

S.C. Administration. The serum chemistry profile of dogs after repeated administration of rHV2 (1 mg/kg, s.c.) is illustrated in Figures 81 to 85. Blood samples were drawn at baseline (pre-rHV2) and 120 minutes (post-rHV2) after each dose, and the sera were analyzed for chemistry parameters. A two-way repeated measures ANOVA was performed, to study the "day" effect and "pre-post" effect, and their interaction, as explained before.

Figure 81 depicts the variations in inorganic phosphorous, albumin, uric acid and total bilirubin at baseline and 120 minutes after each rHV2 administration. No significant differences were seen in all four parameters (two-way repeated measures ANOVA). Similarly, there were no significant fluctuations in serum sodium, chloride, calcium and potassium values, as shown in Figure 82.

There were no significant variations in hepatic enzymes, ALP, ALT and AST, as well as BUN and creatinine after multiple rHV2 administration, as shown in Figure 83. Similarly, Figure 84 illustrates that no significant variations were seen in total CO_2, total protein and γ-GT after daily administration of rHV2 (two-way repeated measures ANOVA).

Figure 85 depicts the changes in LDH, cholesterol, glucose and triglyceride levels with time. There were fluctuations in LDH levels, exhibiting a step-wise decrease at 120 minutes after drug administration on all days, except day 0. No significant day-to-day variations were observed in LDH levels at baseline and 120 minutes ($p = 0.194$). However, there was a

significant "pre-post" effect ($p = 0.06$), as well as a significant interaction ($p = 0.07$). The day-to-day variations in the differences between pre- and post-rHV2 LDH levels were analyzed using a method of contrasts (Wilkinson, 1990; J.Corliss-personal communication). The LDH levels at 120 minutes after rHV2 administration on days 2, 3, 4 and 6 were significantly lower than corresponding baseline values (Figure 85). No significant variations were observed in the other parameters, illustrated in the same Figure (85), i.e. cholesterol, glucose and triglyceride, after multiple, s.c. rHV2 administration.

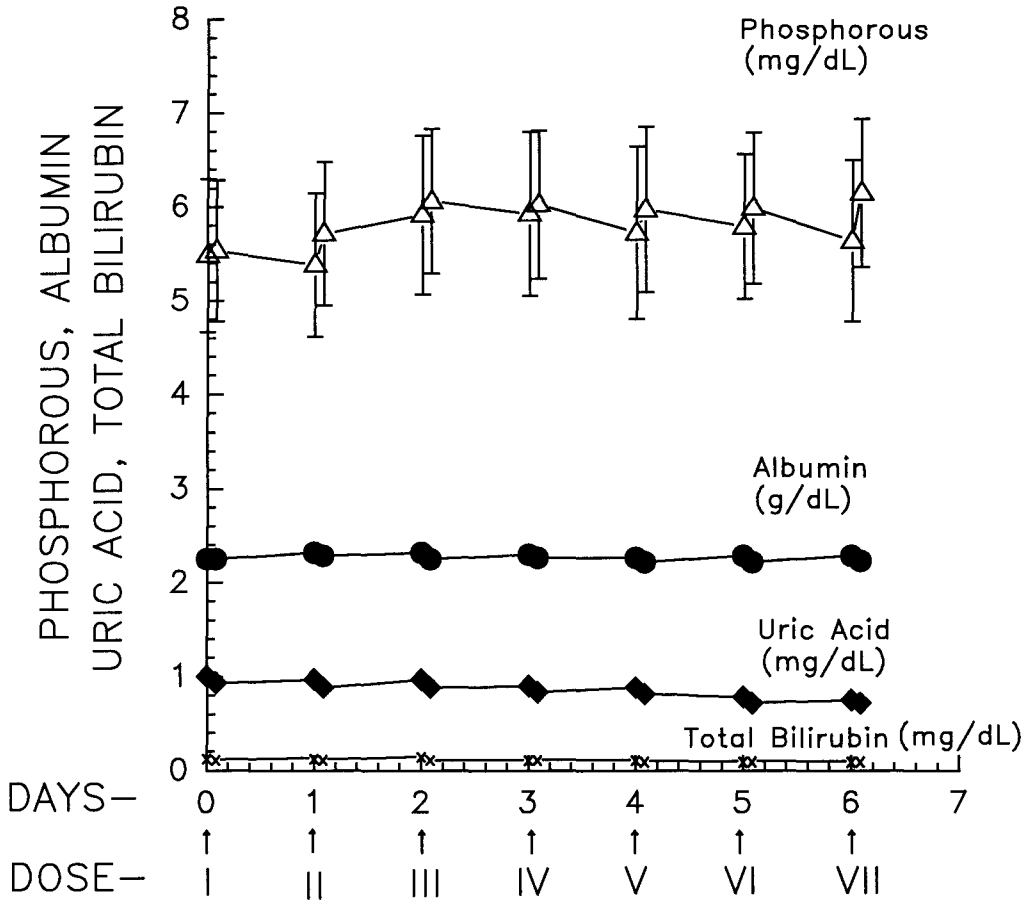


Figure 81. Serum chemistry profile after repeated s.c. administration of rHV2 in dogs (part I). Male, mongrel dogs (n = 6) were injected with 1 mg/kg rHV2 every 24 hours, for a period of one week. Figure shows changes in phosphorous, albumin, uric acid and total bilirubin levels at baseline and 30 minutes after each administration. All data represent the mean determination from 6 dogs ± SEM.

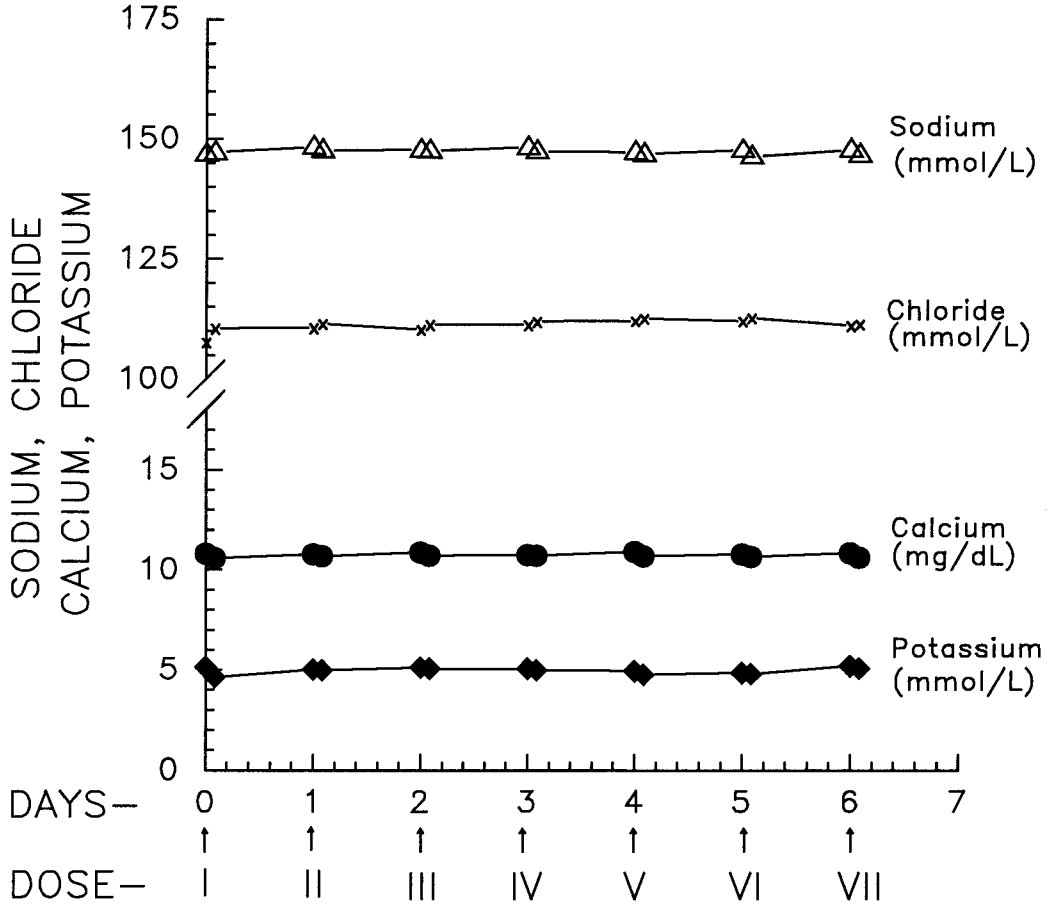


Figure 82. Serum chemistry profile after repeated s.c. administration of rHV2 in dogs (part II). Male, mongrel dogs (n = 6) were injected with 1 mg/kg rHV2 every 24 hours, for a period of one week. Figure shows changes in sodium, chloride, calcium and potassium levels at baseline and 30 minutes after each administration. All data represent the mean determination from 6 dogs ± SEM.

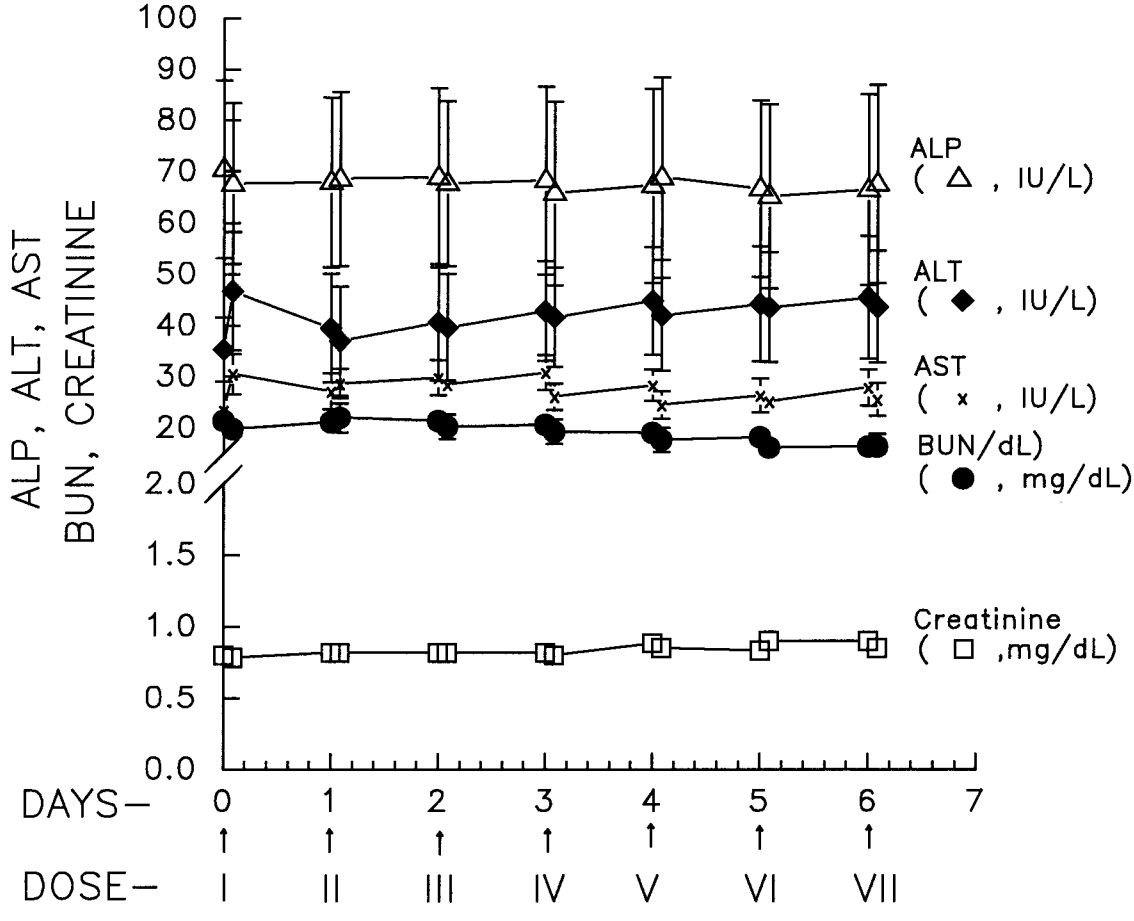


Figure 83. Serum chemistry profile after repeated s.c. administration of rHV2 in dogs (part III). Male, mongrel dogs (n = 6) were injected with 1 mg/kg rHV2 every 24 hours, for a period of one week. Figure shows changes in alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN) and creatinine at baseline and 30 minutes after each administration. All data represent the mean determination from 6 dogs ± SEM.

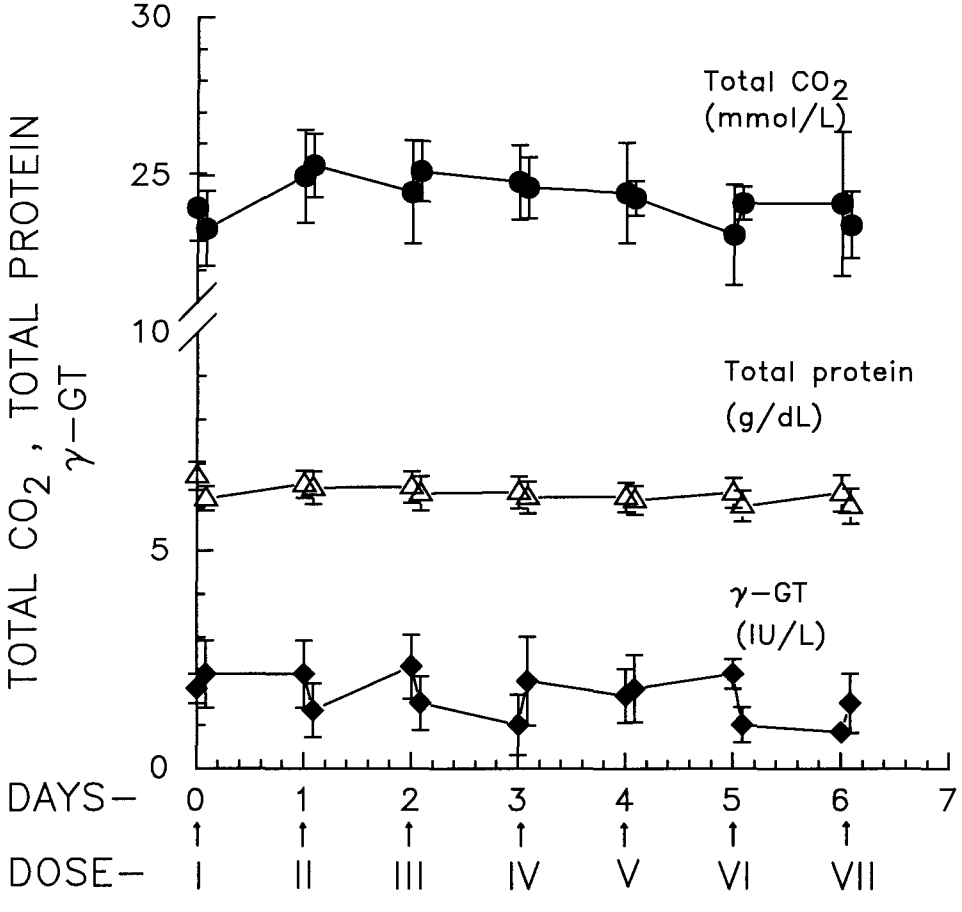


Figure 84. Serum chemistry profile after repeated s.c. administration of rHV2 in dogs (part IV). Male, mongrel dogs (n = 6) were injected with 1 mg/kg rHV2 every 24 hours, for a period of one week. Figure shows changes in total CO_2, total protein and γ-glutamyltransaminase (γ-GT) at baseline and 30 minutes after each administration. All data represent the mean determination from 6 dogs ± SEM.

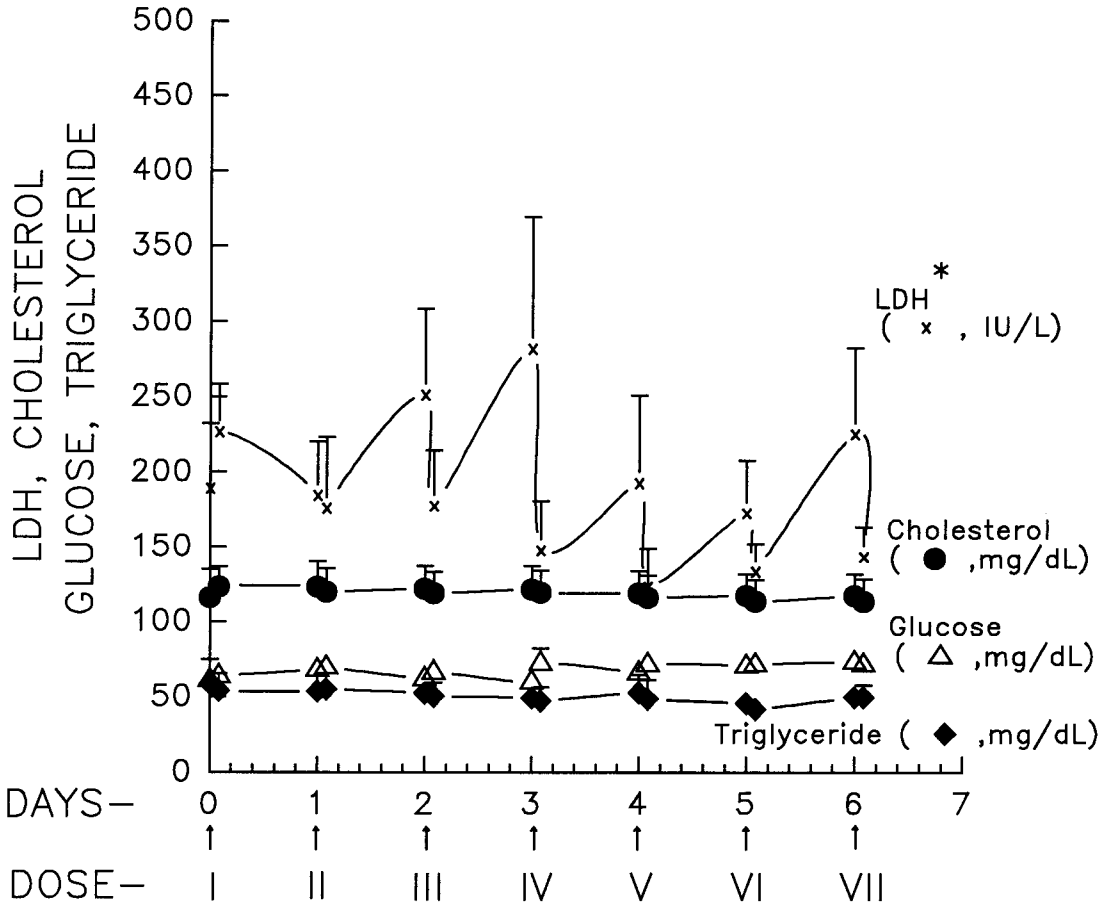


Figure 85. Serum chemistry profile after repeated s.c. administration of rHV2 in dogs (part V). Male, mongrel dogs (n = 6) were injected with 1 mg/kg rHV2 every 24 hours, for a period of one week. Figure shows changes in lactate dehydrogenase (LDH), cholesterol, glucose and triglyceride levels at baseline and 30 minutes after each administration. All data represent the mean determination from 6 dogs ± SEM.

*-$p < 0.1$ (pre and post-rHV2 variation, two-way repeated measures ANOVA).

7b. Hematological Profile after Repeated rHV2 Administration in Dogs

I.V. Administration. Figures 86 to 87 depict the changes in various hematological parameters after i.v. administration of rHV2. Each figure shows the periodic change in different hematological parameters at baseline and 30 minutes after each i.v. dose, up to seven days. Statistical analysis was performed using two-way ANOVA, as discussed before.

The variations in mean cell volume (MCV), hematocrit (HCT), mean cell hemoglobin concentration (MCHC) and mean cell hemoglobin (MCH) levels are shown in Figure 86. No significant differences were seen in MCV, MCHC and MCH values between the times of blood sampling after repeated rHV2 administration. There was a significant decrease in HCT after daily administration of rHV2 ($p = 0.000$, for day effect). Also, there was a significant decrease in HCT at 30 minutes after rHV2 administration, compared to corresponding baseline ($p = 0.002$ for pre-post effect). The difference between pre- and post-rHV2 HCT levels was not significant, from day to day ($p = 0.163$, for the interaction).

Figure 87 illustrates the variations in red cell distribution width (RDW), hemoglobin (HGB) and mean platelet volume (MPV) at different times after multiple rHV2 administration. There were no significant changes in RDW and MPV levels after multiple rHV2 administration. There was a significant decrease in HGB between the various days, as well as between baseline and post-rHV2 HGB levels on each day ($p < 0.05$, two-way repeated measures

ANOVA; $p = 0.129$ for the interaction).

There were no significant alterations in cell count of platelets, WBCs and RBCs, after repeated i.v. administration of rHV2 in dogs, as illustrated in Figure 88.

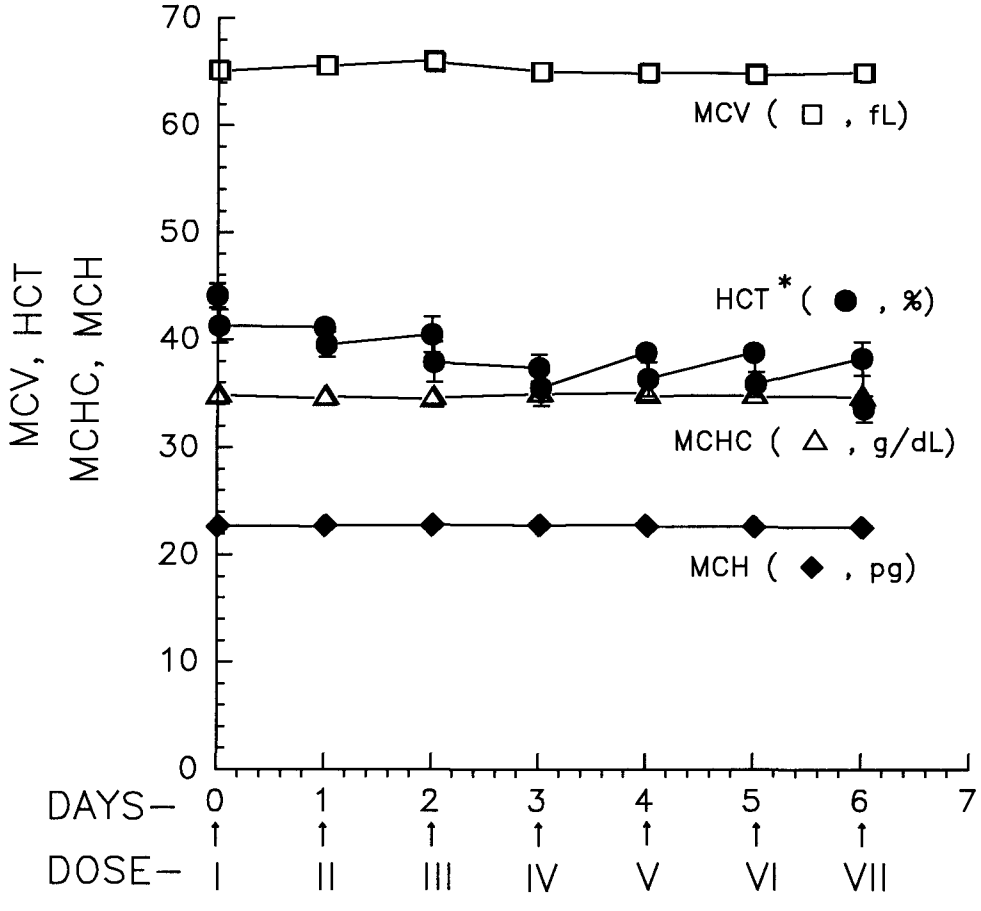


Figure 86. Hematological profile after repeated i.v. administration of rHV2 in dogs (part I). Male, mongrel dogs (n=6) were injected with 1 mg/kg rHV2 every 24 hours, for a period of one week. Figure shows changes in mean cell volume (MCV), hematocrit (HCT), mean cell hemoglobin concentration (MCHC) and mean cell hemoglobin (MCH) at baseline and 30 minutes after each administration. All data represent the mean determination from 6 dogs ± SEM.

*-$p < 0.05$ (significant day-to-day and pre- & post-rHV2 variation, two-way repeated measures ANOVA).

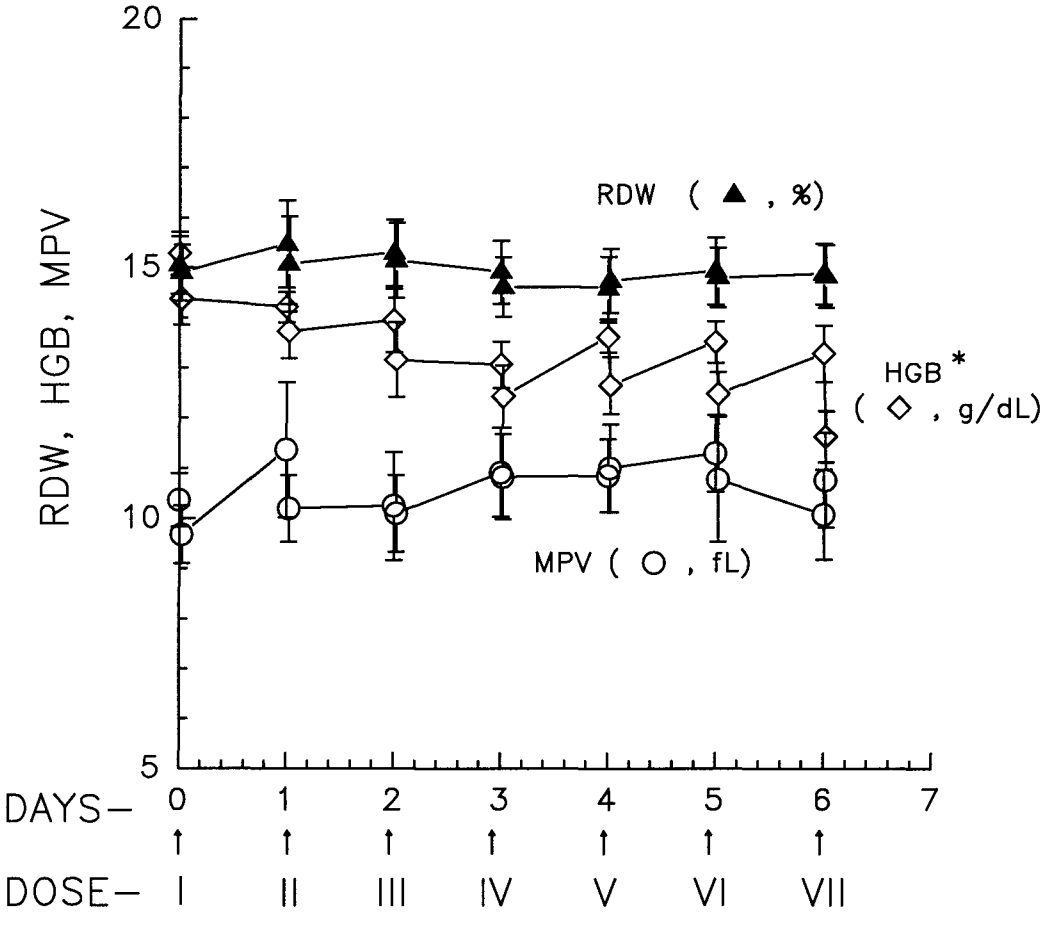


Figure 87. Hematological profile after repeated i.v. administration of rHV2 in dogs (part II). Male, mongrel dogs (n=6) were injected with 1 mg/kg rHV2 every 24 hours, for a period of one week. Figure shows changes in red cell distribution width (RDW), hemoglobin (HGB) and mean platelet volume (MPV) at baseline and 30 minutes after each administration. All data represent the mean determination from 6 dogs ± SEM.

*-$p<0.05$ (significant day-to-day and pre- & post-rHV2 variation, two-way repeated measures ANOVA).

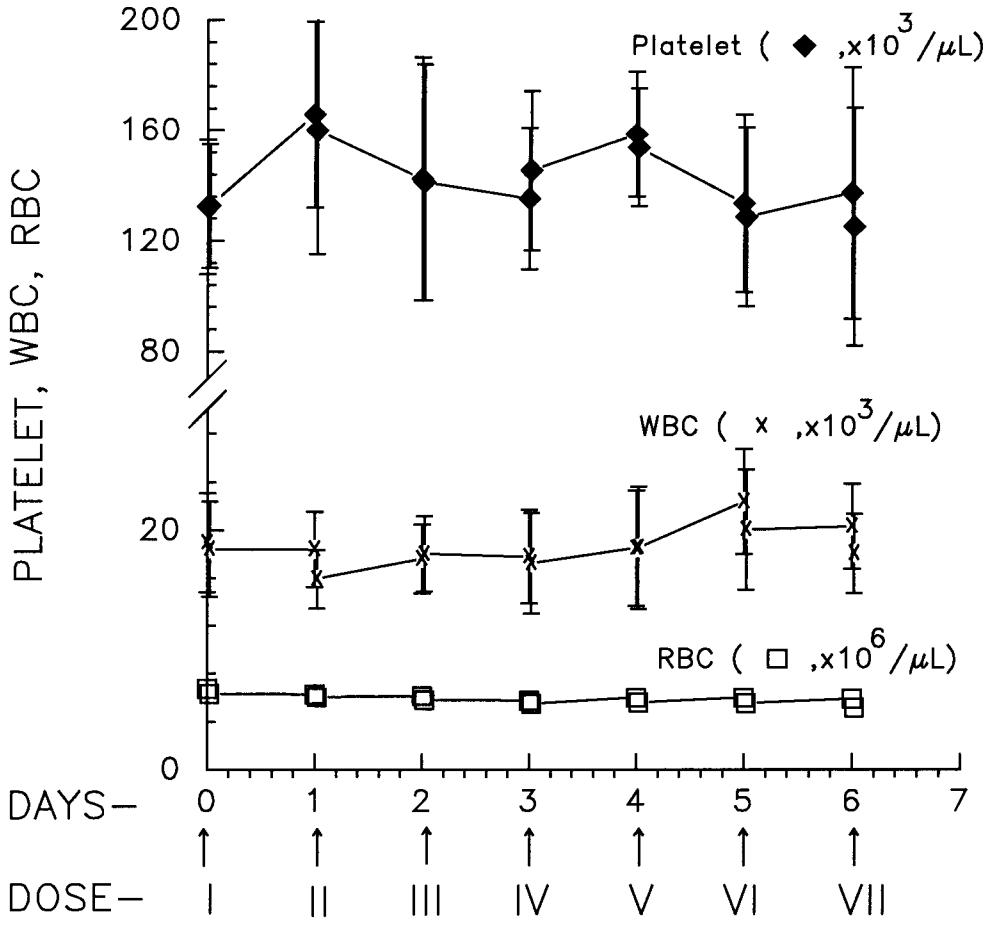


Figure 88. Hematological profile after repeated i.v. administration of rHV2 in dogs (part III). Male, mongrel dogs (n = 6) were injected with 1 mg/kg rHV2 every 24 hours, for a period of one week. Figure shows changes in platelet, RBC and WBC count at baseline and 30 minutes after each administration. All data represent the mean determination from 6 dogs ± SEM.

S.C. Administration. Blood samples were drawn at baseline and 120 minutes after repeated rHV2 administration (1 mg/kg, s.c.) in dogs. The fluctuations in various hematological parameters are described in Figures 89 to 91. Figure 89 exhibits the changes in MCV, HCT, MCHC and MCH after repeated administration. As observed in the i.v. studies, HCT values decreased progressively with time, displaying significant day-to-day variation, as well as significant variation between pre- and post-rHV2 HCT levels on each day ($p < 0.05$, two-way ANOVA; $p = 0.154$ for the interaction term). No significant variations were seen in MCV, MCHC or MCH after multiple rHV2 administration (Figure 89).

The variations in RDW, HGB and MPV are shown in Figure 90. No significant variations were observed in RDW and MPV. However, there was a significant progressive, step-wise decrease in hemoglobin levels ($p < 0.05$, two-way ANOVA; $p = 0.130$ for the interaction). Figure 91 shows that there were no significant variations in blood cell count (RBCs, WBCs and platelets) after repeated s.c. administration of rHV2.

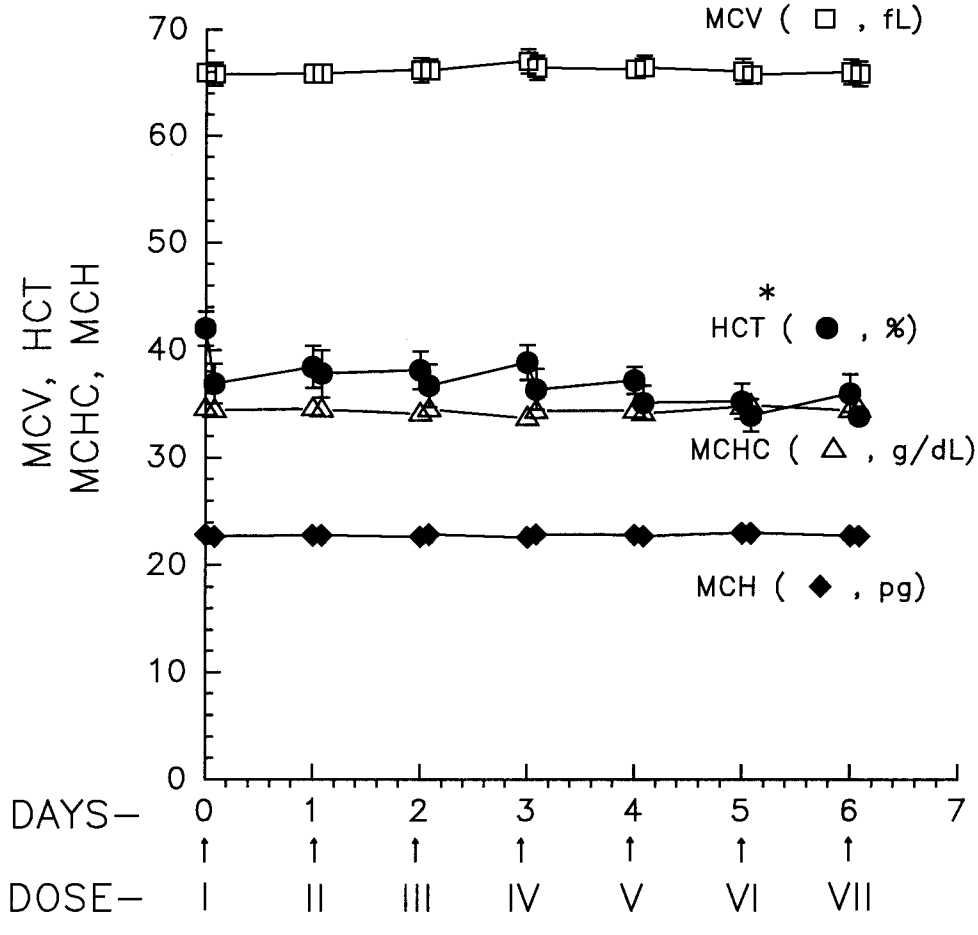


Figure 89. Hematological profile after repeated s.c. administration of rHV2 in dogs (part I). Male, mongrel dogs (n=6) were injected with 1 mg/kg rHV2 every 24 hours, for a period of one week. Figure shows changes in mean cell volume (MCV), hematocrit (HCT), mean cell hemoglobin concentration (MCHC) and mean cell hemoglobin (MCH) at baseline and 30 minutes after each administration. All data represent the mean determination from 6 dogs ± SEM.

*-$p < 0.05$ (significant day-to-day and pre- & post-rHV2 variation, two-way repeated measures ANOVA).

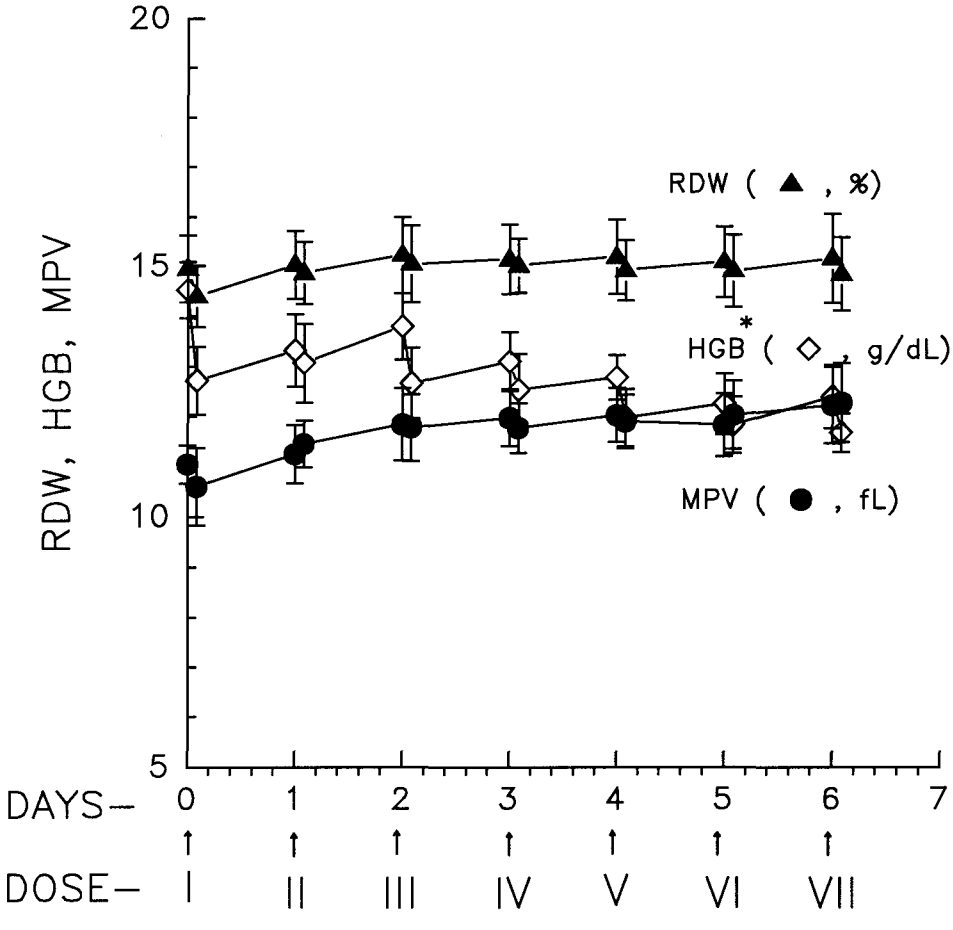


Figure 90. Hematological profile after repeated s.c. administration of rHV2 in dogs (part II). Male, mongrel dogs (n = 6) were injected with 1 mg/kg rHV2 every 24 hours, for a period of one week. Figure shows changes in red cell distribution width (RDW), hemoglobin (HGB) and mean platelet volume (MPV) at baseline and 30 minutes after each administration. All data represent the mean determination from 6 dogs ± SEM.

*-$p < 0.05$ (significant day-to-day and pre- & post-rHV2 variation, two-way repeated measures ANOVA).

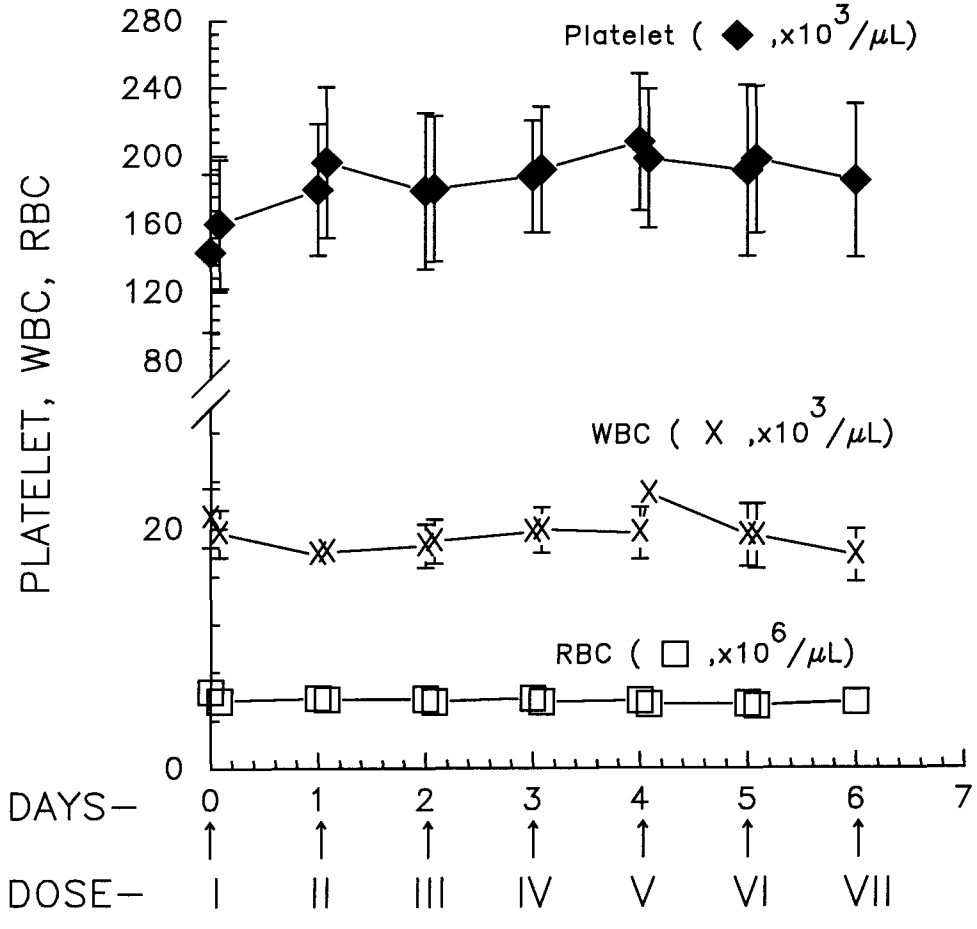


Figure 91. Hematological profile after repeated s.c. administration of rHV2 in dogs (part III). Male, mongrel dogs (n = 6) were injected with 1 mg/kg rHV2 every 24 hours, for a period of one week. Figure shows changes in platelet, RBC and WBC count at baseline and 30 minutes after each administration. All data represent the mean determination from 6 dogs ± SEM.

CHAPTER V

DISCUSSION

In Vitro Studies

Hirudin is a 65 or 66 amino acid polypeptide that was isolated from the salivary glands of medicinal leeches. Recombinant forms of the native protein isoforms are currently available. Hirudin and rH variants are highly potent inhibitors of thrombin (K_i = 50 pM). rHs are undergoing investigation for use as a prophylactic and therapeutic antithrombotic agent after deep venous thrombosis and unstable angina, during coronary bypass surgery, and to prevent restenosis after percutaneous transluminal coronary angioplasty.

In this dissertation, the pharmacology of recombinant hirudin variant 2 (rHV2) has been studied, using pharmacokinetic and pharmacodynamic methods. Specific experimental designs have been developed and executed to characterize the pharmacokinetic and pharmacodynamic properties of rHV2 in different animal species, such as dogs, rabbits and rats.

1. Construction of Concentration-Response Curves after *In Vitro* Supplementation of rHV2 in Plasma Systems

Assay dependent variations in the responses of antithrombotic drugs in laboratory monitoring methods have been known for some time. Besides the mechanisms of activation in individual assays, the dilution of recombinant hirudin in samples due to the addition of assay reagents may contribute significantly to differences in observed end points. Thus, the current assay methods used are not equivalent and provide results that are distinct from one another. In this dissertation, a systematic study on the direct comparison of various assays in reference to the assay matrix effect and adjustment of final assay concentration of rHV2, has been carried out. The purpose of concentration-response studies reported in this dissertation is to provide an objective assessment of the relative effects of hirudin in different assays with special reference to assay conditions and final concentrations.

A profile of *in vitro* anticoagulant and antithrombin activities of rHV2 was obtained in pooled animal plasma preparations in parallel with *ex vivo* coagulation and anti-IIa assays from *in vivo* experiments in rats, rabbits and dogs. In addition, the *in vitro* anticoagulant and antithrombin activity was investigated after supplementation of rHV2 in blood bank plasma (BBP) in an effort to relate the results from animal studies with human studies.

Selection of coagulation and anti-IIa assays. The coagulation tests used to evaluate the *in vitro* anticoagulant activity of rHV2 included activated partial

thromboplastin time (APTT- OT and Dade), thrombin time (TT), and calcium thrombin time ($Ca^{+2}TT$).

APTT is used to evaluate abnormalities in the intrinsic pathway of blood coagulation. It has been the conventional clotting method used to monitor heparin therapy (Racanelli, 1990; Camici and Evangelisti, 1993) and has been used to evaluate hirudin anticoagulant activity, as well (Chesebro *et al.*, 1991a; 1991b). Heparin exhibits multiple sites of action on coagulation enzymes (II_a, X_a and XII_a) and its anticoagulant action is mediated by antithrombin-III (AT-III) and heparin cofactor-II (HC-II) (Ofusu *et al.*, 1981, 1982, 1985). Heparin has been shown to release tissue factor pathway inhibitor (TFPI) from endogenous sites, but there is no evidence of TFPI being released by hirudin (Fareed, 1994). Recent clinical studies with rH have utilized APTT as the method to monitor hirudin activity (Antman, 1994; Gusto IIa investigators, 1994). However, the validity of the use of APTT to predict hirudin activity has not been established. Theoretically, hirudin would prolong APTT as it interferes with the final pathway of coagulation by inhibiting thrombin. It may be speculated that APTT is prolonged by hirudin due to the inhibition of thrombin mediated feedback activation of factors V and VIII, thereby interfering with the intrinsic system.

Recent reports have debated the use of APTT as a suitable assay method to monitor rH therapy (Iyer *et al.*, 1990; Lefkovitz and Topol, 1994). rH is a relatively weak inhibitor of thrombin generation when compared with

heparin (Kaiser *et al.*, 1992). Hence, a clotting assay that would directly target the thrombin inhibitory activity of rH would be more appropriate, such as the TT or the modified Ca^{+2}TT, discussed in the following sections. Another important factor to be considered is the variability in the various reagents available to perform the APTT assays (Poller, 1980; Howarth, 1993). Tripodi *et al.* (1993) observed that there were significant, reagent based differences in the APTT responses in hirudinized normal human plasma using ten different APTT reagents. In addition, APTT responses have been reported to be highly sensitive to changes in storage time and temperature (Ho and Wu, 1991).

Two different APTT reagents were used in the experiments reported in this dissertation: APTT (OT) and APTT (Dade). The APTT (OT) reagent is composed of rabbit brain phospholipids with micronized silica as an activator and hence, has an insoluble contact activation matrix. The APTT (Dade) reagent contains cephalin extracted from dehydrated rabbit brain in ellagic acid. Ellagic acid is a soluble activator of the contact system, and it is stronger than micronized silica. Hence, the APTT (OT) assay is more sensitive than the APTT (Dade). In all *in vitro* and *ex vivo* analyses using the two APTT reagents, the baseline clotting values using the Dade reagent was at least 50% lower than that obtained with the OT reagent. Furthermore, the results from most *in vitro* and *ex vivo* analyses indicate variable profiles of APTT (OT) and APTT (Dade) in different species, as discussed later.

A more specific coagulation assay to measure the direct thrombin inhibitory activity of rH is TT. However, the clotting response using this assay exhibited very little concentration dependency when rH was supplemented in normal rabbit plasma (NRP) (Figure 12), as well as in other plasma systems such as normal human, monkey, and dog plasma (Iyer *et al.*, 1990). The TT (10 U/mL) values reached beyond 300 seconds which is the upper limit of sensitivity for this assay using the Fibrometer®, at a concentration as low as <1 pM/mL (Figure 12), due to the strong affinity of rH for thrombin.

To obtain more definite end points in the TT assay, the thrombin reagent was modified by reconstituting α-thrombin in 0.025M $CaCl_2$ solution to obtain calcium thrombin reagent. This assay has been previously validated and has been used to monitor hirudin's anticoagulant activity in various plasma preparations (Iyer *et al.*, 1990). It was observed that using an appropriate balance of thrombin and $CaCl_2$ content in the reagent, a well defined, sigmoidal concentration-response curve could be obtained, using Ca^{+2}TT (10 U/mL) and rHV2 supplemented NRP (Figure 12).

In addition to the coagulation assays, a biochemically defined amidolytic anti-IIa assay that measures the release of pNA due to the action of the enzyme (thrombin) on the substrate (Spectrozyme®TH,) in the presence of the inhibitor (rHV2), was used. This method has been employed by previous investigators to study the antithrombin effects and pharmacokinetics of rH (Greissbach *et al.*, 1985; Markwardt *et al.*, 1988).

Concentration-response curves. The baseline clotting times using Ca^{+2}TT (10 U/mL) were similar between rabbit, dog and blood bank plasma. APTT (Dade) values did not significantly differ between species, either. However, the baseline APTT (OT) in rabbit plasma was significantly greater than that obtained with dog or blood bank plasma (57.05/NRP vs. 18.6/NDP vs. 27.56/BBP, $p<0.05$, one way ANOVA followed by Tukey test). This observation agrees with previous results with baseline APTT (OT) in rabbits (Racanelli, 1990). However, the reason for this finding is not clear. It has been shown that several clotting factors (II, VII, VIII, IX, XI and XII) were actually 2 to 28 fold higher in rabbits compared with humans (Walenga, 1987). However, the functionality of clotting factors in rabbits in comparison to human or other species is not clear at this time. Furthermore, one cannot rule out the possibility of functional inhibitory effects due to endogenous inhibitors like antithrombin III. The results from baseline coagulation parameters emphasize the importance of species differences in clotting profiles. It is critical to use concentration-response curves from the same species during extrapolation of *in vitro* results to *ex vivo* experiments.

The alignment of concentration-response curves in the plasma preparations studied (except NRatP) was very similar (Figures 13 to 15). Ca^{+2}TT (10 U/mL) was the most sensitive assay to monitor rHV2 in NRP, NDP, and BBP, exhibiting linear increases in responses at very low concentrations i.e. 1 and 10 pM/mL. Therefore, this assay can be used to

monitor concentrations of rHV2 in the respective plasma in the above range, translating to original plasma concentrations of 10 to 100 ng/mL.

APTT (OT) might be useful to monitor rHV2 concentrations in the range of about 10 to 250 pM/mL, which corresponds to original plasma concentrations of 0.2 to 3.0 μg/mL. APTT (Dade), on the other hand, exhibited linear ranges varying with individual plasma systems. The linear ranges for APTT (Dade) were about 150 to 250 pM/mL in NRP, 100 to 250 pM/mL in NDP and 50 to 250 pM/mL in BBP, corresponding to an average range of 2 to 5 μg/mL, in terms of original plasma concentrations.

The linear range of the chromogenic anti-IIa assay was between 0.12 and 1.2 μg/mL in rabbit, dog and blood bank plasma in terms of original rHV2 concentrations. The concentration-response curve for anti-IIa assay in NRatP was shifted to the right when compared with that obtained in NRP, NDP and BBP, thereby shifting the linear range to 1.2 - 12.0 μg/mL. Higher concentrations of rHV2 were required to produce equivalent thrombin inhibitory activity in rat plasma. The reason for this finding is not known at this time. Rat blood was found to be hypercoagulable compared to other species. Glass activated (37^{o}C) clotting times in rats have been reported to be 2-5 minutes (Ringler and Dabich, 1979) when compared to 8.6 minutes in dogs, 5 minutes in rabbits and 7.5 minutes in humans (Didisheim *et al.*, 1959). Rats have been shown to have a much higher platelet count (1000,000 to 1275,000/μL, Schalm *et al.*, 1975) than rabbits (750,000/μL, Kozma *et al.*,

1974), dogs (250,000/μL, Schalm *et al.*, 1975) and humans (293,000/μL, Didisheim *et al.*, 1959).

In the absence of a true maximal response in most of the coagulation assays, a slope analysis was carried out in the linear portions of each individual concentration-response curve. The slope represents the rate of change in intensity of responses with respect to variations in rHV2 concentrations in the linear range. The dimensions of slopes were seconds/pM/mL for the coagulation assays and % inhibition of thrombin/pM/mL for the chromogenic anti-IIa assay. Evaluation of the slopes indicated that the Ca^{+2}TT (10 U/mL) responses exhibited much higher slopes in comparison to the two APTT assays in rabbit, dog and blood bank plasma, as shown in Table 5. This meant that the Ca^{+2}TT (10 U/mL) was more sensitive to small changes in rHV2 concentrations than the two APTT tests. This can be explained by the different biochemical mechanisms involved in the two clotting assays: thrombin is supplemented in the Ca^{+2}TT (10 U/mL) assay, while thrombin is generated in the APTT assay. With regard to the anti-IIa assay results in NRatP, the response exhibited much lower sensitivity to changes in rHV2 concentrations than that observed in the other plasma systems.

The results from the concentration-response curves generated *in vitro* point toward the need to choose an appropriate assay method to monitor rH. Walenga *et al.* (1991a) have projected a circulating rH level of 5 to 10 μg/mL

for therapeutic anticoagulation. In the case of Ca^{+2}TT (10 U/mL), dilutions will be necessary to obtain final assay concentrations in the linear range. The use of this modified thrombin time is most logical, taking into account the mechanism of action of hirudin. Newer studies are warranted to establish a standardized calcium-thrombin reagent to decrease variability and improve the linear range of the assay. In the case of APTT, the assay may not be as sensitive and as appropriate as a direct, thrombin based coagulation assay. To emphasize, APTT reagents from the same source must be used for effective comparison studies. The biochemical, chromogenic anti-IIa assay is reproducible, but might require dilutions to monitor rH in therapeutic concentrations.

Species dependence in concentration-response curves. A wide physiological variation on the hemostatic profile of various species has been noted by several investigators (Didisheim *et al.*, 1959; Kozma *et al.*, 1974; Schalm *et al.*, 1975). Major differences are also obvious in the plasma composition of human, rabbit, rat and dog plasma. Since this dissertation addressed the pharmacological effects of rHV2 in various species, a detailed analysis of the effects of rHV2 in various plasma preparations was made. Besides the species dependent variations, assay dependent variations within the same species introduced complications in the interpretation of the results. Until now, there is no reliable method available to monitor the effects of rH in

pharmacological or clinical studies. In this dissertation, an attempt was made to validate the utility of some of the conventional assays such as APTT. Furthermore, since major differences among the APTT reagents were noted, a thrombin titration method to measure antithrombin activity of rH has been developed.

The *in vitro* studies to determine the concentration-response curves in plasma from different species resulted in providing assay, and species specific concentration-response curves. With the exception of the amidolytic anti-IIa assay, all of the other assays provided distinct concentration-response curves and sensitivity ranges for rH in the rabbit, human and dog plasma systems. However, even in this assay, NRatP revealed a wide difference. These studies suggest that a cross-species, universal method to monitor rH is not feasible at this time. However, with proper standardization, both optimized clot based and amidolytic methods can be used to determine the anticoagulant and antithrombin activities of rH.

The *in vitro* analysis of anticoagulant and antithrombin properties of rHV2 using various assay methods were performed in parallel with *ex vivo* analysis from *in vivo* experiments. However, the influence from various physiologic mechanisms including uptake and disposition mechanisms, feedback mechanisms and circadian rhythms as well as individual-to-individual physiological variations must be taken into consideration while relating *in vitro* results with *ex vivo* measurements of rHs.

2. Determination of Specific Activity of rHV2 using a Thrombin Titration Method

The determination of potency of biological activity for various antithrombotic drugs still remains a major controversy. A standard method to evaluate the potency of hirudin has not been established to date. The thrombin titration method utilized in this dissertation is an extension of the chromogenic anti-IIa assay developed to monitor hirudin activity in plasma (Greissbach *et al.*, 1985, J. Amiral-personal communication). The method is based on the 1:1 stoichiometric reaction between hirudin and thrombin (Markwardt, 1970). The described method is simple, rapid and highly reproducible with a coefficient of variation of 2.1% (Iyer and Fareed, 1995). The average specific activity obtained of rHV2 used in this research was found to be ≈ 15,873 ATU/mg (Figure 17). This was about 9% more than that claimed by the manufacturer which could be due to variations in additives such as fillers and preservatives. The three individual experiments were performed over a period of one week and no significant differences were found between the individual specific activities (Table 6). It is to be noted that the thrombin used during titration must be of known, high purity. The thrombin utilized in this study was composed of 95.72% of α-thrombin. It may be noted that the shape of the curve (Figure 17) suggests that the rHV2-thrombin interaction may not be of a 1:1 binding ratio. The thrombin titration method has been agreed upon by the World Health Organization (WHO) and

International Society of Thrombosis and Haemostasis (ISTH) affiliate (Longstaff *et al.*, 1993) as the reference method for potency evaluation of various hirudins.

The thrombin titration method can be used to determine specific activity of incoming batches of rH for their antithrombin activity prior to *in vitro* as well as *in vivo* experiments in animal and human subjects. The method can be used to compare the potencies of different batches of rH, to relate to the potency of a reference standard, to determine variations in potency of hirudin during storage, and as part of routine stability studies in the development of hirudin as an antithrombotic agent.

3. Determination of Protein Content of rHV2 using the Δ 215-225 Method

Since homogeneous protein solutions of rHV2 were used, a spectrophotometric method was employed to measure protein content. The recovery of the protein content, as determined in rHV2 solutions of 25, 50 and 100 μg/mL was found to be 109.6, 112.4 and 102.2%, respectively, with an average of 108%. The linear range for the Δ 215-225 method is 10 to 100 μg/mL (Chaykin, 1966). This finding indicated that there was no loss of protein due to shipment, storage conditions or other degradation in the batch of rHV2 used.

In Vivo Studies

1. Assessment of Antithrombotic Profile of rHV2 using a Modified Jugular Vein Stasis Thrombosis Model in Rabbits - A Dose-Ranging Study

The dose-ranging experiments on the antithrombotic actions of rHV2 were designed to demonstrate the relevance of different dosages on the antithrombotic actions of this agent. In this investigation, both intravenous and subcutaneous protocols were included. The results from this study were taken into consideration for the selection of a dosage of rHV2 for the time course experiments, using intravenously and subcutaneously administered rHV2.

For the evaluation of *in vivo* antithrombotic actions of rHV2, a well-defined and established model of rabbit stasis thrombosis was used employing activated prothrombin complex concentrates (FEIBA®) as the thrombogenic challenge (Fareed *et al.*, 1985). This model has been extensively used in the evaluation of several antithrombotic and anticoagulant agents such as heparin, low molecular weight heparins, dermatan sulfate and site-directed thrombin inhibitors (Fareed *et al.*, 1985; Walenga, 1987; Hoppensteadt, 1989; Racanelli, 1990; Bacher *et al.*, 1993). However, in the current investigation, an additional modification was made by extending the stasis time to 20 minutes on one jugular vein segment, in contrast to 10 minute stasis times for both segments in some of the earlier studies. This modification provided an additional parameter to compare the antithrombotic actions of rHV2 in

relatively stronger thrombogenic conditions.

I.V. Administration. In comparison to the saline treated control groups, rHV2 produced a dose dependent antithrombotic effect in both 10 minute and 20 minute stasis studies. In relation to the 10 minute stasis, the antithrombotic effect of rHV2 was relatively weaker when a 20 minute end point was used. This is primarily due to the stronger thrombogenic environment produced by the longer stasis time. Both end points exhibited a dose dependent antithrombotic activity of rHV2, with an apparent ED_{50} of 9 μg/kg for the 10 minute stasis, and approximately 11.5 μg/kg for the 20 minute stasis time. This is consistent with the pathophysiology of this model, as the 20 minute stasis time results in a stronger thrombogenic environment in which, stasis, along with activated coagulation factors, is capable of producing a relatively stronger thrombogenic environment. At the 25 μg/kg dosage, a complete absence of clot formation indicated total inhibition of the thrombogenic process at the 10 minute stasis interval. On the other hand, a residual thrombogenic activity was evident in the 20 minute stasis time results, where a 25% clotting response persisted. These results clearly indicated a dose, and experimental condition dependent antithrombotic effect of rHV2. These findings also point toward the fact that the antithrombotic action of thrombin inhibitors, such as rHV2, are dependent on experimental conditions and the type of the thrombogenic trigger used.

In order to relate the observed antithrombotic effects of rHV2 with the *ex vivo* tests which are currently used to measure the anticoagulant/ antithrombotic actions of rHV2, several methods were employed. Formulations of APTT using a particulate activator {APTT (OT)}, and an ellagic acid based soluble activator {APTT (Dade)} were used to test the anticoagulant effects of rHV2 in this study. With APTT (OT), at 6.25 and 12.5 μg/kg, a moderate, dose dependent, though insignificant increase was noted in the 5 minute "post-drug" samples (Figure 19). At 25 μg/kg, no increase in clotting times was evident. When the three doses were compared with saline control, no significant differences were observed. Blood samples were collected 6 minutes after the administration of thrombogenic challenge, in addition to the post-drug samples. In the "post-FEIBA®" samples, at all doses, no significant difference was evident. Interestingly, the clotting times obtained after FEIBA® administration were significantly shortened. This significant shortening of the clotting time after FEIBA® administration supports the hypothesis that FEIBA® produces a thrombogenic environment capable of producing some neutralization of the anticoagulant actions of rHV2. In comparison to the APTT (Dade) studies (Figure 20), a wider scatter in the data was seen with this reagent. This indicates that different APTT reagents provide varying results. Thus, for uniform results, a single APTT reagent must be used.

With APTT (Dade), the *ex vivo* analysis of the group of rabbits treated

with 6.25 μg/kg did not reveal any significant differences between saline and rHV2 treated groups (Figure 20). This was interesting, as at the 10 minute stasis time, using the same dosage, there was a significant ($p<0.05$) reduction in clot scores compared with controls (Figure 18). Hence, while anticoagulation was not detectable using this APTT (Dade) reagent, a marked inhibition of clot scores was evident in the 6.25 μg/kg treatment group. At the 12.5 μg/kg dosage, a >30% increase in APTT (Dade) was noted, compared with baseline value. At the 25 μg/kg dosage, although a further prolongation of APTT (Dade) was noted, it was not proportional to the administered doses of rHV2. The APTT increase at this dosage was about 2-fold that of saline treated control rabbits.

Although no difference in APTT (Dade) was noted after the 6.25 and 12.5 μg/kg doses of rHV2 between the samples taken prior to, and after FEIBA® administration, a significant shortening of APTT (Dade) was observed after the 25 μg/kg dose. This is consistent with the observation that FEIBA® is capable of producing a thrombogenic environment.

To analyze the direct antithrombin effects in the clot-based assays, the thrombin based tests, TT and Ca^{+2}TT were performed. At a 6.25 μg/kg dose, a slight prolongation of TT (10 U/mL) was noted (Figure 21). At 12.5 and 25 μg/kg, the clotting times reached >300 seconds. In the post-FEIBA® samples, no significant differences were noted in all three dosage groups when compared with corresponding post-drug samples. In the Ca^{+2}TT (10

U/mL) assay, a dose dependent anticoagulant effect with a definite end point was obtainable at all dosages (Figure 22). The corresponding elevation of the clotting time was shorter in this group when compared to the TT (10 U/mL), as discussed earlier. A significant shortening of clotting time was noted in the post-FEIBA® samples in the 12.5 and 25 μg/kg groups, when compared with corresponding 5 minute post-drug samples. The fact that a progressive prolongation of the clotting times with definite end points was obtained with the Ca^{+2}TT (10 U/mL) reagent suggests that this reagent is better for monitoring of the anticoagulant effects of rHV2.

A chromogenic substrate based amidolytic assay capable of solely detecting the antithrombin activity of rHV2 was employed. A dose dependent inhibition of thrombin was noted with this assay (Figure 23). At 25 μg/kg, almost 80% thrombin inhibitory activity was observed. Interestingly, the post-FEIBA® samples collected at different dosages exhibited marked decreases when compared to the corresponding post-drug samples. These results, once again, reinforce the notion that FEIBA® produces a strong thrombogenic environment where rHV2 is neutralized as measured by a decreased inhibition of thrombin.

In this investigation, a parallel assessment of the anticoagulant actions of rHV2 was made using different clotting methods and a specific chromogenic anti-IIa assay to compare with the observed antithrombotic actions of rHV2. This provided a comparative assessment of the relative value

of some of these *ex vivo* tests in the monitoring of the antithrombotic actions of rHV2. The APTT (Dade), Ca^{+2}TT (10 U/mL) and anti-IIa assays provided relatively consistent dose dependent effects. All three assays also detected the thrombogenic effects of FEIBA®. Since, rHV2 represents a monospecific thrombin targeting agent, these results provide supportive evidence on the use of antithrombin assays in the assessment of *ex vivo* activity of rHV2.

S.C. Administration. rH has been reported to exhibit a short half-life after i.v. administration (Bichler *et al.*, 1988; Richter *et al.*, 1988). Previous studies have been performed using the subcutaneous route and have demonstrated an increase in the biological residence time of rHV2 (Iyer *et al.*, 1993a). The dose-ranging study to evaluate the antithrombotic activity employed this additional route (s.c.) to compare and contrast the *in vivo* and *ex vivo* activity of rHV2 after the two routes of administration.

There was a dose dependent increase in the antithrombotic activity of rHV2 after s.c. administration at both the 10 minute and 20 minute stasis times (Figure 24). Clot-scores of approximately "+3" and "+4" were obtained in the saline treated group at 10 and 20 minutes, respectively, which were consistent with the results from the i.v. studies. A dose dependent antithrombotic effect was observed after rHV2 doses of 125, 250 and 375 μg/kg. In the groups with 10 minute stasis times, results compiled at 250 and 375 μg/kg indicated a complete inhibition of clot formation. However, at the

125 μg/kg, only a 60% inhibition of clot formation was noted. Relatively weaker antithrombotic activity of rHV2 was observed at the 20 minute stasis times than that obtained at the corresponding 10 minute stasis times ($p < 0.05$ after saline, 125 and 250 μg/kg of rHV2). This finding confirms the fact that a 20 minute stasis time results in a relatively stronger thrombogenic environment, which was also observed in the i.v. studies. ED_{50} values were calculated to be approximately 90 and 220 μg/kg, after 10 and 20 minute stasis times, respectively. At 375 μg/kg, rHV2 exhibited a complete absence of clot formation at 10 minutes and a slight residual clotting response at 20 minutes.

A series of *ex vivo* coagulation tests were performed in the s.c. studies to relate to the observed antithrombotic effects in the stasis thrombosis model, similar to the i.v. studies. In the particulate based APTT (OT) assay, there was a dose dependent, significant increase in coagulation times with increasing rHV2 administration (Figure 25). In addition, there was a significant decrease in clotting activity after administration of FEIBA® when compared with the 5 minute post-drug samples. In contrast, with the APTT (Dade) assay, there was no clear dose dependency of the clotting times as the dose of rHV2 was increased (Figure 26). In fact, none of the three doses of rHV2 increased the clotting times significantly beyond baseline values. There appeared to be a lesser thrombogenic effect of FEIBA® as was evident from the apparent almost equal clotting times in the post-FEIBA® samples when

compared with those obtained at 5 minutes after rHV2 administration. A possible explanation for this observation may be the presence of trace amounts of protein C in FEIBA® (Fareed *et al.*, 1984).

The results from APTT (Dade) and APTT (OT) tests after s.c. administration provided inconsistent data in comparison to the corresponding results obtained after i.v. administration. APTT (Dade) exhibited a more clear gradation of responses with different escalating doses when compared to APTT (OT), in the case of i.v. administration. On the other hand, after s.c. administration, the APTT (OT) assay was a better test to effectively differentiate between the three rHV2 doses and saline, when compared with APTT (Dade). While the reason for this apparent discrepancy is not clear, it may be possible that the rHV2 molecule is enzymatically modified after s.c. administration, possibly leading to different binding characteristics of rHV2 to thrombin, and hence leading to differential responses to the coagulation process initiated by varying APTT reagents.

After s.c. administration, the thrombin reagent based coagulation tests, TT (10 U/mL) and Ca^{+2}TT (10 U/mL), were performed in a manner identical to the i.v. studies. Interestingly, there was no demonstrable difference in both the TT (10 U/mL) and the Ca^{+2}TT (10 U/mL) responses between the three subcutaneous doses of rHV2 (Figures 27 and 28, respectively). In the case of TT (10 U/mL), there was a steep increase in clotting times beyond 300 seconds with the lowest dose, i.e 125 μg/kg, when compared to saline. With

Ca^{+2}TT (10 U/mL), there were definite end points in the post-drug and post-FEIBA® samples from some of the rabbits in the lower two doses but the coagulation times exceeded the 300 second limit of sensitivity after administration of 375 μg/kg of rHV2 (Figure 28). Furthermore, there was no significant reduction in the coagulation times in the samples obtained after FEIBA® administration in comparison to those obtained after rHV2 administration, using both tests. Addition of $CaCl_2$ solution to the conventional thrombin reagent did not contribute significantly in facilitating the coagulation process initiated by FEIBA®, in this set of results.

The specific antithrombin activity of rHV2 was also determined by using the amidolytic anti-IIa assay, as described earlier. The results from this assay after s.c. administration provided a clear, dose dependent prolongation of thrombin inhibitory activity with rHV2 administration in increasing doses (Figure 29). A 90% inhibition of thrombin was observed in plasma samples after administration of the 375 μg/kg dose. It was interesting to note that there was no significant difference in the antithrombin activity between the post-FEIBA® and corresponding post-drug samples after administration of each dose of rHV2.

In contrast to the results from the i.v. studies, the APTT (OT) and the anti-IIa assay provided more consistent results with the *ex vivo* results after s.c. administration of rHV2. This finding, again, can be explained by possible chemical or structural alteration of rHV2 after s.c. administration, producing

differential responses with various *ex vivo* coagulation tests. The possible role of subcutaneous barriers in the bioavailability of rHV2 after this route of administration is discussed elsewhere in this dissertation.

A dosage of 375 μg/kg of rHV2 was selected for subsequent time course studies. It is important to point that a 38% thrombin inhibitory activity was obtained two hours after rHV2 administration in the lowest dose of 125 μg/kg (Figure 29). At this dosage, the corresponding clot score was "+1" (Figure 24) and the corresponding APTT (OT) value (Figure 25) was approximately 90 seconds (baseline ≈ 65 seconds). The 125 μg/kg rHV2 dose was strong enough to increase the clotting times close to or beyond 300 seconds in both the TT (10 U/mL) and Ca^{+2}TT (10 U/mL) assays. Even though this 125 μg/kg dose did produce considerable antithrombotic activity at the 10 minute stasis time, it was only partially effective at the 20 minute stasis time (clot score of "+2"). Hence, successive increments of rHV2 doses beyond this dosage to 250 and 375 μg/kg were made. A clearly detectable antithrombotic activity was obtained with 375 μg/kg at both 10 and 20 minutes in the stasis thrombosis experiment.

The results from the s.c. studies support the use of *ex vivo* antithrombin assays such as the chromogenic thrombin substrate based assay to monitor the antithrombotic activity of rHV2. The results from this assay correlated well with the observed antithrombotic actions of rHV2. Thus, the amidolytic anti-IIa assay may be useful in the monitoring of antithrombotic activity of rHs.

2. Assessment of Hemorrhagic Activity of rHV2 using a Modified Rabbit Ear Blood Loss Model

The hemorrhagic profile of rHV2 was investigated using a modified ear blood loss model, as described by Cade *et al.*, 1984. This model has been used previously and has also been shown to yield reproducible results (Hoppensteadt, 1989; Racanelli, 1990; Bacher *et al.*, 1993). The mean RBC loss from saline treated control rabbits was 0.026 X 10^9 RBCs/L, which agreed with previous results using the same model (Racanelli, 1990; Bacher *et al.*, 1993).

I.V. Administration. Intravenous administration of rHV2 in escalating doses exhibited a dose dependent increase in hemorrhagic effect (Figure 30). However, hemorrhagic responses were significant only with the higher doses of rHV2. When used in the experimentally determined antithrombotic i.v. dose of 25 μg/kg of the agent, the RBC loss was comparable to that obtained with saline treated control rabbits.

There was an apparent decrease in RBC loss after injection of the 2.5 mg/kg dose of rHV2 (Figure 30). Nonetheless, this decrease in bleeding response after the 2.5 mg/kg dose, was not significantly different from the response after 1 mg/kg, both at 5 and 15 minutes. Hence, it may be concluded that the maximal limit of the hemorrhagic effect was attained after the 1 mg/kg dose of rHV2. The apparent dose to produce 50% of maximal toxicologic (hemorrhagic) response (TD_{50}) was calculated to be 0.7 and 0.8

mg/kg, at time periods of 5 and 15 minutes, respectively.

S.C. Administration. Similar to the i.v. studies, a dose dependent increase in bleeding was observed with increasing s.c. doses of rHV2, in the modified rabbit ear blood loss model (Figure 31). There was comparable intensity of bleeding between experimentally determined antithrombotic s.c. dose of rHV2 (375 μg/kg) and saline treated control rabbits.

The blood loss after administration of 5 mg/kg was used as the maximal response as further increases of rHV2 beyond 5 mg/kg may become toxic. The TD_{50} for the bleeding response after s.c. administration of rHV2 was calculated to be 2.8 and 0.8 mg/kg, at time periods of 1 and 3 hours, respectively.

Protective Index for rHV2. Using the calculated ED_{50} values for antithrombotic activity, and TD_{50} values for hemorrhagic activity of rHV2 in rabbits, a pharmacological term, known as protective index (P.I.) was calculated. P.I. is given by the ratio: TD_{50}/ED_{50} (Kokate *et al.*, 1994). It is a measure of relative toxicity of a drug, and has been used previously to study the hemorrhagic indices of other antithrombotic drugs such as heparin and dermatan sulfate (Santoro *et al.*, 1992).

The P.I.s were obtained from the dose-ranging studies for antithrombotic activity at 5 minutes and hemorrhagic activities at 3 hours after rHV2 administration in rabbits. The calculated P.I. values for rHV2 were as

follows:

I.V.: P.I. = TD_{50}/ED_{50} = 700/9 = 78

S.C.: P.I. = TD_{50}/ED_{50} = 800/90 = 9

It is clear that rHV2 has very high safety index, when used intravenously. The reason for the lower safety after s.c. administration is the 10-fold increase in ED_{50} values after s.c. administration, for the antithrombotic activity. Jeske (1995) found that the P.I. for unfractionated heparin was 17, using the same models of venous thrombosis and blood loss in rabbits. Santoro *et al.* (1992) observed that the P.I. values for i.v. heparin and dermatan sulfate were 1 and 4, respectively, using venous thrombosis model and tail transection bleeding time in rats.

It may be concluded that rHV2 is a relatively safe antithrombotic agent, exhibiting little/no hemorrhagic activity at antithrombotic doses in normal rabbits. However, at the same time, caution needs to be exercised during use of this agent in critical situations, as was evident from the results from the recent clinical trials on target patient population (Antman, 1994; Gusto IIa investigators, 1994). The relevance of these findings in other species may have to be carefully established because of the significant variations in the hemostatic profile in each species.

3. Evaluation of Pharmacokinetics and Time Course of Antithrombotic Activity after rHV2 Administration in Rabbits

The purpose of the time course study in rabbits using the jugular vein stasis thrombosis model was to integrate pharmacokinetics of rHV2 with pharmacodynamic effects, as measured using antithrombotic actions of this agent. The time dependency of both *in vivo* and *ex vivo* antithrombotic and anticoagulant effects of rHV2 was studied in rabbits. rHV2 was used at an i.v. dose of 25 μg/kg and a s.c. dose of 375 μg/kg.

I.V. Administration. At the 10 minute stasis interval, a complete and sustained *in vivo* antithrombotic activity was observed up to about 30 minutes after i.v. administration of rHV2 (Figure 32). However, at the 20 minute stasis interval, this activity was decreased at each corresponding circulation time of rHV2. This observation agrees with the results from the dose-ranging experiments in rabbits using the same model, where a stronger thrombogenic environment was evident at the 20 minute stasis interval.

A gradual increase in clot scores was obtained at the 10 minute interval with increasing circulation times of rHV2. The clot scores at the 20 minute stasis time remained steady at approximately +4 from 15 minutes to 3 hours, indicating almost no antithrombotic effect of rHV2, under this condition. The ultimate physiological mechanism of action of hirudin has been predicted to be the inhibition of thrombin generation (Fenton II *et al.*, 1991). Recent reports, however, indicate that rH is not as effective as heparin in the

inhibition of thrombin generation (Kaiser *et al.*, 1992). Stasis for 20 minutes may have allowed for a greater amount of thrombin generation than that may have been generated at the 10 minute stasis time, thereby antagonizing the antithrombotic effect of rHV2.

A sigmoidal relationship was found when the results from the stasis thrombosis model were integrated with pharmacokinetics of rHV2 in rabbits (Figure 37). This indicated a classical biological response with a sigmoidal shape with increasing concentrations of rHV2. A significant correlation coefficient of 0.93 was obtained in the linear portion of the sigmoidal curve.

The relationship between measured plasma rHV2 concentrations and the observed antithrombotic responses after i.v. administration of rHV2 was further studied, using a sigmoidal E_{max} model (Holford and Sheiner, 1982). The sigmoidal E_{max} model is a modification of the classical E_{max} model, and accounts for the shape of the concentration-effect relationship (Holford and Sheiner, 1982). It is a modification of the Hill equation that was proposed to describe the association of oxygen with hemoglobin (Hill, 1910).

The sigmoidal E_{max} may be described as follows:

$$E = \frac{E_{max} \cdot C^n}{EC_{50}^n + C^n}$$

, where E is the antithrombotic effect at a concentration, C. E_{max} and EC_{50} are the maximal effect and the concentration required to produce 50% of maximal effect, respectively. n is a parameter that describes the shape of the curve. Figure 92 illustrates this relationship, where the reciprocal of clot scores was used to express antithrombotic activity. The simulated responses correlated significantly with the observed responses with a correlation coefficient of 0.99 (Figure 92). The E_{max}, EC_{50}, and n values obtained for the simulated curve were 21.5 ± 0.4 (clot-score of ≈ 0.047), 0.238 ± 0.003 μg/mL, and 4.4 ± 0.3, respectively (mean ± SEM). These values matched the observed the observed E_{max} and EC_{50} values, closely.

The sigmoidal curve after intravenous rHV2 administration in rabbits represents crucial information on the plasma concentration-antithrombotic effect relationship of rHV2. This model indicates a direct relationship between rHV2 concentrations measured in the plasma and the observed antithrombotic responses. The equation for this model suggests that it may be likely that the rHV2-thrombin interaction may not be of a 1:1 binding ratio. This model may be used to predict magnitudes of antithrombotic responses after measuring plasma rHV2 concentrations. The significant correlation between the actual values and predicted responses may be due to the fact that the effector site of the rHV2 is represented by the sampling compartment.

The sigmoidal E_{max} relationship between plasma rHV2 concentrations and antithrombotic responses may be affected by the probable conversion of

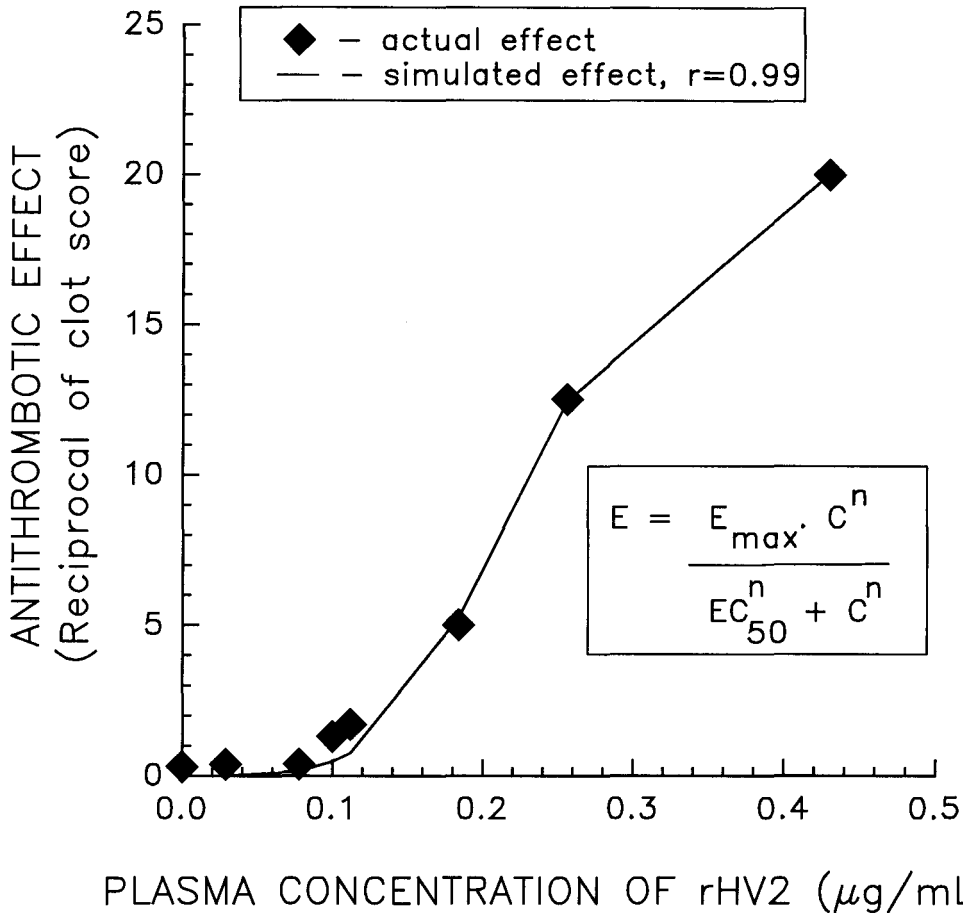


Figure 92. The sigmoidal E_{max} model describing the relationship between antithrombotic effects of rHV2 and measured plasma rHV2 concentrations. The antithrombotic activity was measured using a modified jugular vein stasis thrombosis model in rabbits (n = 5). Plasma concentrations were measured using the competitive ELISA method. E_{max} = 21.5 ± 0.4, EC_{50} = 0.238 ± 0.003 μg/mL and n = 4.4 ± 0.3.

rHV2 to active or inactive metabolites. Another factor that can affect this relationship may be the possible formation of antibodies to recombinant forms of hirudin, when administered repeatedly.

With regard to the *ex vivo* assays, the time course of $Ca^{+2}TT$ (10 U/mL) (Figure 34) and amidolytic anti-IIa assay (Figure 35) were almost superimposable with the time course of plasma concentrations of rHV2 (Figure 36). This observation was substantiated by the fact that significant correlations were obtained between the plasma rHV2 concentrations and the $Ca^{+2}TT$ (10 U/mL) ($r=0.87$, Figure 39) as well as the thrombin inhibitory activity of rHV2 ($r=0.94$, Figure 38). The time course of APTT (OT) and APTT (Dade) assays correlated poorly with pharmacokinetics of rHV2, compared to the above tests (Figure 38). Therefore, a direct relationship between pharmacokinetics and pharmacodynamics of rHV2 was observed after i.v. administration of rHV2 using the $Ca^{+2}TT$ (10 U/mL) and chromogenic anti-IIa assay.

The pharmacokinetics of rHV2 in rabbits was analyzed using standard non-compartmental analysis (Gibaldi, 1984; Gibaldi and Perrier, 1982; Rowland and Tozer, 1989). The advantages and limitations of compartmental modeling techniques have been of major interest to investigators (Wagner, 1975; Yates, 1978; Fleishaker and Smith, 1987; Rescigno and Beck, 1987). In this dissertation, non-compartmental analysis based on statistical moment theory was used, as "the distinction between a drug that follows single-

compartment model and one that follows two-compartmental kinetics is often not clear" (Gwilt, 1990). Moreover, it was found that different subjects within the same species exhibited different multicompartment kinetics, similar to previously reported results on other drugs (Gibaldi, 1984). Hence, model independent parameters such as clearance and volume of distribution were estimated using the statistical moment theory (Yamaoka *et al.*, 1978).

The pharmacokinetic parameters determined in this study agreed with previous results in rabbits using a chromogenic thrombin substrate (Chromozym®TH) assay (Nowak, 1991; Markwardt *et al.*, 1982). Terminal elimination half-life ($t_{1/2}$) after single dose i.v. administration was about 55 minutes using the competitive ELISA method, which agrees with results published on the natural hirudin by Markwardt *et al.* (1982, 62 minutes), and on recombinant hirudin by Nowak (1991, 70 minutes).

The plasma rHV2 concentration-time curve (Figure 36) indicated an instantaneous distribution phase of rHV2 followed by an elimination phase with a rate constant of 0.76 hr^{-1}. The volume of distribution (V_d) was found to be approximately 137 mL/kg. This indicated a significant extravascular component in the volume of distribution of rHV2 as normal blood volume in rabbits has been reported to be about 55-57 mL/kg (Kozma *et al.*, 1974). This may also explain the lower than expected C_{max} value of 0.75 μg/mL at 3 minutes. A significant amount of this agent may have instantaneously distributed to the extravascular compartments before the first sampling time

of 3 minutes.

S.C. Administration. The time course of antithrombotic activity and pharmacokinetics of rHV2 were evaluated in rabbits in a similar manner, as performed in the i.v. studies. Detailed information on the pharmacokinetics and pharmacodynamics of recombinant hirudin after s.c. administration in rabbits is not available to date.

There was a major difference in the time course of physiological antithrombotic activity of rHV2 after s.c. administration, depending on the time period of stasis induced in the experimental conditions. Administration of a 375 μg/kg dose of rHV2 coupled with a 10 minute stasis period, resulted in an almost complete antithrombotic state (Figure 40). On the other hand, increasing the time of stasis to 20 minutes resulted in a progressive increase in antithrombotic activity up to 2 hours with a decline in activity, thereafter. A possible explanation for this finding may be that the dose of rHV2 employed was considerably strong to maintain an antithrombotic state up to 9 hours after administration and to oppose the thrombogenicity provided by the stasis period of 10 minutes. In addition, the clot score grading system employed, i.e. 0 to +4, provides a general gradation of antithrombotic activity. If it were possible to further differentiate the antithrombotic responses between a clot score of 0 and that of +1 at various levels, a more clear, step-wise gradation of antithrombotic activity would have been made available. If such a grading

system was available, it would be possible to detect differences in the antithrombotic activity of rHV2 at various levels after different circulation times.

Increasing the stasis time to 20 minutes resulted in a pattern of antithrombotic effect that was more appropriate for s.c. administration, with a peak, absorption phase and elimination phase (Figure 40). A sharp peak in *in vivo* activity was observed at 2 hours using a 20 minute stasis time. In contrast, a sustained, steady maximal response was observed between approximately 1 and 4 hours, using *ex vivo* tests such as $Ca^{+2}TT$ (10 U/mL) (Figure 42) and chromogenic anti-IIa assay (Figure 43). This can again be explained by the fact that the responses in the stasis thrombosis model are graded on an ordinal scale, while the responses in coagulation tests, anti-IIa assay and plasma rHV2 concentrations are measured on an interval scale.

When the *in vivo* antithrombotic activity in rabbits was related with plasma concentrations of rHV2 (Figure 45), a relationship similar to a counter-clockwise hysteresis loop was obtained when the points of observation were joined in a sequence of time (Holford and Sheiner, 1981; 1982). According to Holford and Sheiner (1982), "this form of hysteresis is characteristic of a delay in equilibration between plasma drug concentration and the concentration of active substance at the effect site". A characteristic lag phase was observed to elicit maximal antithrombotic activity. In Figure 45, as the time progressed from 0.5 to 2 hours, increasing concentration of rHV2

was "available" in the plasma, thereby increasing the antithrombotic activity. There was a steep decline in antithrombotic response from 2 to 2.5 hours and 3 hours, even though the corresponding plasma concentrations were approximately 0.3 and 0.26 μg/mL, respectively. This can again be explained by the ordinal nature of the clot score grading system.

A clot score of approximately +3.5 at 4 hours was obtained even though the plasma rHV2 concentration was at its maximum (0.38 μg/mL). This may be explained by the possible degradation of rHV2 after s.c. administration to products that are functionally inactive or less active, but maintain their immnunogenicity and hence, are detected by the ELISA method. The fate of hirudin after extravascular administration is not clear to this date. Enzymatic degradation of the hirudin molecule into products that may or may not be active is a good possibility. Information on the possible interaction of hirudin with components and enzymes in the endothelium is not clear. Pizzo *et al.* (1990) have shown that recombinant hirudin forms a complex with factor X_a and displaces factor X_a from endothelial binding sites. Previous preliminary studies in dogs with rHV2 have indicated an increased bioavailability and decreased sensitivity in coagulation tests and chromogenic anti-IIa assay after s.c. administration when compared with equal dose of i.v. administration (Iyer *et al.*, 1993a).

With regard to the *ex vivo* coagulation tests, no time dependency was exhibited by APTT (Dade) assay after s.c. administration of rHV2 (Figure 41).

APTT (OT) and APTT (Dade) responses correlated poorly (r = 0.60 and 0.19, respectively) when compared with corresponding responses after i.v. administration (Figure 46). As observed in the i.v. studies, the time course of Ca^{+2}TT (10 U/mL) (Figure 42) and chromogenic anti-IIa assay (Figure 43) were almost identical to the plasma concentration-time curve (Figure 44). Ca^{+2}TT (10 U/mL) responses correlated less significantly (r = 0.73) with pharmacokinetics after s.c. administration when compared to i.v. administration (r = 0.87). The chromogenic anti-IIa assay correlated significantly with plasma concentrations after s.c. administration (r = 0.89).

As mentioned earlier, the pharmacokinetics of rH has not been studied extensively after s.c. administration in rabbits. The bioavailability after s.c. administration of rHV2 was found to be approximately 45%, in these studies (Table 3). The total clearance rate was found to be identical to that found after i.v. administration, i.e. 104 mL/hr/kg. The volume of distribution (V_d) was about the same as blood volume in rabbits, i.e. 58 mL/kg. This may be due to the decreased exposure of the drug across the subcutaneous barrier.

In addition, the terminal elimination half-life ($t_{1/2}$) was only half of that found after i.v. administration. The $t_{1/2}$ after i.v. administration is a better indication of the elimination characteristics of a drug than extravascular administration. The increase in terminal elimination rate (k_e) after s.c. administration may be an example of a reverse "flip-flop" model (Byron and Notari, 1976) where the estimate of elimination is driven by the estimate of

absorption. According to Byron and Notari (1976), the negative slopes of log linear plots of plasma concentration-time curves following extravascular drug administration may not represent reasonable estimates for the first order elimination rate constant. Absorption after s.c. administration is a complicated process involving the different layers of the skin and the presence of several enzymes. A flip-flop phenomenon or "vanishing exponential terms" in pharmacokinetic analysis of extravascular data may be observed due to the problems and uncertainties associated with the definition and differentiation of absorption and disposition profiles of a drug (Chan and Gibaldi, 1985).

Due to such disparity in the $t_{1/2}$ values of rHV2 between the two routes of administration, the mean residence times (MRT) were used as a better indication of the biological residence time of rHV2. The MRT indicates the time taken for the elimination of 63.2% of a drug from the body; it is the non-compartmental counterpart of the biological half-life (Gibaldi and Perrier, 1982). Clearly, the MRT after s.c. administration was at least five times higher after i.v. administration. From the k_e values after i.v. and s.c. administration, a significant portion of the MRT must be due to the absorption phase.

A critical evaluation of the results from the i.v. and s.c. experiments in rabbits indicated that the biological residence time may be increased by using the s.c. route. However, caution needs to be exercised while using *ex vivo* coagulation tests to monitor rHV2 during s.c. administration due to an

apparent, comparatively poor correlation of some of the tests with plasma concentrations. The fate of rHV2 in terms of metabolic degradation after s.c. administration still remains to be delineated.

4. Determination of Pharmacokinetic Characteristics and *Ex Vivo* Antithrombin Activity of rHV2 in Rats

The purpose of this study was to profile the pharmacokinetics of rHV2 in rats after administration of escalating doses via two routes of administration (i.v. and s.c.). Furthermore, the pharmacokinetics was integrated with *ex vivo* thrombin inhibitory activity of rHV2, using the amidolytic anti-IIa assay.

I.V. Administration. There was a dose dependent increase in plasma concentrations of rHV2 with escalating doses in rats (Figure 49). The peak plasma concentration (C_{max}) and area under the plasma concentration-time curve ($AUC_{0-\infty}$) increased with the administration of each increasing dose, in a linear fashion (Table 8). Hence, it may be assumed that rHV2 exhibits linear pharmacokinetics in rats. At the same time, the total clearance rate and volume of distribution did not vary with increasing doses (Table 8). This finding agrees with previous results using escalating doses of rH in human, male subjects (Meyer *et al.*, 1990). Therefore, the use of rH may be more advantageous than heparin in clinical indications, as heparin has been known to exhibit non-linear pharmacokinetics (Bjornsson and Levy, 1979a, 1979b; Emanuele, 1987). This may ease the dose adjustments that may be necessary

during monitoring of subjects on rH therapy.

It must be mentioned that the V_d after the lowest dose was lower (0.94 L/kg) with the lowest dose, when compared with that obtained with the higher two doses (1.7 and 1.8 L/kg). However, appropriate statistical analysis could not be done, as the pharmacokinetic parameters listed in Table 8 (as well as those in Table 9/rat and Tables 17 and 18/dog) were calculated from the composite plasma concentration-time curve. The parameters exhibited a wider scatter in rats, than those obtained after escalating rHV2 administration in dogs (discussed later).

Extravascular distribution of rHV2 was evident from the V_d parameters, as the blood volume in rats is reported to be $\approx$ 64 mL/kg (Ringler and Dabich, 1979). The elimination half-life ($t_{1/2}$) in the terminal portion of plasma concentration-time curve did not alter after administration of each dose, with an average $t_{1/2}$ of about 22 minutes. This was found to be approximately three times smaller than corresponding observation (64 minutes) made by Markwardt *et al.* (1988a)., using recombinant hirudin in rats. In this study, the $t_{1/2}$ after i.v. administration was about 22 minutes after all three doses, with at least 24 rats in each treatment (dose) group. However, the methods used for the determination of plasma concentration of rH in the two studies are different: chromogenic anti-IIa assay versus the competitive ELISA method. In addition, Markwardt *et al.* (1988a) used recombinant hirudin variant 1 (rHV1) in Wistar rats, while this study employed the use of variant

2 of rH in Sprague-Dawley rats.

With regard to the *ex vivo* antithrombin activity, rHV2 exhibited proportional increases in anti-IIa activity, with increasing i.v. doses (Figure 50). Significant correlations (>0.90) was obtained with plasma rHV2 concentrations after administration of all three i.v. doses of rHV2.

S.C. Administration. Similar to the i.v. studies, there was a dose dependent increase in C_{max} values after s.c. administration (Figure 51, Table 9). The terminal elimination half-life was about an hour after all three s.c. doses of rHV2. Most pharmacokinetic parameters led to the conclusion that rHV2 exhibits linear pharmacokinetic after s.c. administration.

It has been shown that the clearance of rH in rats is largely extra-renal, when compared to other species, after i.v. (bolus and infusion) and s.c. administration of rHV2 (Richter *et al.*, 1988). Previous results from the Hemostasis Research Laboratories, Loyola University Chicago, also confirm the decreased renal clearance of rHV2 in rats (cumulative urinary excretion, CUE, after i.v. dose = 5% of dose, data not shown). It may be possible that with increasing s.c. administration, there is a saturation of enzymatic degradation of rHV2 in organs such as the liver. More studies are needed to investigate the exact mechanisms of non-renal clearance of rHV2 in species such as the rat.

The results from the *ex vivo* antithrombin activity (Figure 52) after s.c.

administration exhibited good correlations with corresponding plasma rHV2 concentrations determined by the ELISA method.

In conclusion, it may be stated that rHV2 exhibited linear pharmacokinetics in rats on the basis of proportional increases in peak concentrations and area under the curve values, after increasing doses. This is substantiated by the similar elimination half-lives, total clearance and volumes of distribution after all three doses via both routes of administration.

5. Evaluation of Renal Function after rHV2 Administration in Rats

It has been proposed that recombinant hirudin would be an ideal drug for use as a prophylactic antithrombotic agent in the prevention of deep venous thrombosis, unstable angina and during cardiopulmonary bypass surgery (Fareed *et al.*, 1989; Fareed *et al.*, 1991b; Johnson, 1994). Hence, it was important to determine the effect of rH on renal function. This becomes particularly important with the knowledge that rH has been shown to be primarily excreted unchanged (up to 92%) by the kidney (Markwardt, 1991b). To investigate the effect of rHV2 on renal function, a ^{3}H-inulin clearance study was performed in rats at 24 hours, after i.v. and s.c. administration of rHV2.

Inulin is a polysaccharide that is not metabolized in the body, and is uniquely excreted from the body only by glomerular filtration without tubular reabsorption or secretion (Alving and Miller, 1940). Hence, the measurement

of inulin clearance has been shown to be a highly reliable method to evaluate renal function via estimation of glomerular filtration rate (GFR) (Alving and Miller, 1940; Ferguson *et al.*, 1950; Barnard *et al.*, 1955; Prescott *et al.*, 1991). In this dissertation, a pharmacokinetic approach has been employed to measure the rate of inulin clearance (Cl_{in}), using time-decay curves of radioactivity in plasma after a single i.v. bolus injection of ^{3}H-inulin. This method has been used previously and has been shown to reflect GFR, closely (Luke *et al.*, 1991; Prescott *et al.*, 1991). This method was simple and rapid, permitting Cl_{in} rates to be measured from plasma time-decay curves, thereby eliminating repeated collections of urine samples from subjects.

The results from the ^{3}H-inulin clearance studies in rats indicated that rHV2 did not alter renal function at a dose of 0.5 mg/kg, i.v., and 1.0 mg/kg, s.c., when compared with corresponding saline treated controls. The plasma time-decay curves of radioactivity were almost identical in the four treatment groups (Figure 53). The calculated Cl_{in} rates were approximately 1.10 and 1.36 mL/min/100g body weight, after i.v. and s.c. administration of rHV2 respectively (Table 7). The corresponding values obtained after saline administration were 1.3 and 1.46 mL/min/100g body weight, respectively. These values for Cl_{in} closely matched the reported value for normal GFR in rats, i.e., approximately 1.02 mL/min/100g body weight (Ringler and Dabich, 1979).

Concern over the delayed clearance of rHs, resulting in extended

anticoagulant effects and bleeding complications, have been expressed, recently (Lefkovitz and Topol, 1994). This is primarily due to renal compromise in a select group of patients, such as the elderly. The pharmacokinetics of hirudin may be altered in patients with varying degrees of renal compromise. The ^{3}H-inulin clearance studies reported here clearly indicate that rHV2 itself does not produce any renal compromise. Thus, a patient's own predisposing factors may be responsible for the observed sustained anticoagulant and bleeding complications in certain clinical trials (Antman, 1994; Gusto IIa Investigators, 1994).

5a. Serum Chemistry Profile in Rats after Administration of rHV2

Serum chemistry parameters were analyzed prior to, and 24 hours after rHV2 administration, in order to evaluate any acute effects of rHV2 on renal, cardiac, liver and other general functions in rats.

I.V. Administration. All baseline serum chemistry markers evaluated (except lactic dehydrogenase/LDH) were within reported, normal ranges in rats, as shown in Tables 11 and 12 (Ringler and Dabich, 1979; Lang, 1993b). Evaluation of liver function enzymes such as alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT), indicated that a 0.5 mg/kg dose of rHV2 did not alter hepatic function and integrity, when compared with saline treated controls. Hence, rHV2 administration did not induce jaundice, hepatobiliary disease or hepatitis

(Zimmerman, 1984). Normal values of γ-GT in the rHV2 treated group indicated an absence of hepatobiliary disease. The significant decrease in γ-GT in the saline treated group at 24 hours may be a result of stress, induced during the blood sampling procedure. The normal values of albumin (ALB) and total protein (T PROT) showed no defects in protein metabolism by the liver. Normal total bilirubin (T BIL) indicated absence of jaundice (Zimmerman, 1984).

Plasma cholesterol level was unchanged after rHV2 administration, when compared to saline treated control values. The significant decrease in triglyceride (TRIG) level after administration of rHV2, when compared to corresponding baseline, may also be due to the stress induced during collection of blood samples. This decrease in TRIG level was found in the saline treated groups, as well. Hyper- or hypoglycemia was not observed after rHV2 administration, as illustrated by normal glucose levels at baseline and 24 hours after rHV2 administration.

Serum LDH levels in rats from all groups were much higher than reported normal values of 15 IU/L (Loegering *et al.*, 1974). However, according to Ringler and Dabich (1979), normal LDH values vary widely with each assay method used. Rat serum LDH has been shown to increase following bacterial infection (Mitruka and Jonas, 1969), administration of adrenergic stimulants (Marmo *et al.*, 1973) and exercise (Loegering, 1974; Ringler and Dabich, 1979). In this study, there was no evidence of any

infection, nor were the rats administered with any adrenergic agents. It was observed that baseline LDH levels were similar between rats acclimated to normal housing and metabolic cages (data not shown). Furthermore, the LDH level was higher than expected at baseline, even before saline or rHV2 treatment, as shown in Table 9. The reason for the slight, yet significant ($p<0.1$), lowering of LDH 24 hours after saline treatment is not known.

There were no changes in serum electrolyte values such as those of sodium (Na^+), potassium (K^+) and chloride (Cl^-), after i.v. administration of rHV2 (Table 9), indicating normal renal regulation of electrolytes (Murphy *et al.*, 1984). In addition, normal kidney function was illustrated by serum creatinine (CREAT) and blood urea nitrogen (BUN) levels, which were also unchanged between saline and rHV2 treated groups. Calcium (Ca^{+2}) and inorganic phosphorous (PHOS) levels were indicative of normal calcium and phosphorous homeostasis (Table 9).

It is not clear if the slight increase in total CO_2 value at 24 hours was due to the administration of rHV2, or a result of hypoventilation or ventilation/ perfusion inequalities (Murphy *et al.*, 1984), due to the inhalation of halothane.

In summary, rHV2 did not significantly alter most serum biochemical markers, except total CO_2 and triglycerides, at 24 hours, when used in an i.v. dose of 0.5 mg/kg.

S.C. Administration. As observed in the i.v. studies, all baseline serum chemical markers (Tables 13 and 14) were within normal ranges reported (Ringler and Dabich, 1979; Lang, 1993b). Recent work has shown that rH (15 mg, s.c., b.i.d.) exhibited a lesser influence on liver enzymes, such as ALT, AST, γ-GT, and ALP, when compared with unfractionated heparin (5000 IU, s.c., t.i.d.), in patients undergoing total hip replacement (Lindbratt *et al.*, 1995).

In general, there were no significant alterations in most parameters after s.c. administration of rHV2 (1.0 mg/kg), compared with saline treated controls. This indicated normal cardiac, renal and kidney functions after rHV2 administration. However, there was a significant decrease in TRIG level in rHV2 treated rats at 24 hours, when compared with baseline, in agreement with the i.v. results (Table 13). Moreover, a slight, yet insignificant, reduction in TRIG was also observed in the saline treated control group.

A significant decrease in LDH levels was observed after rHV2 administration, which again, may be due to the stress of blood sampling procedure (Table 14). A moderate, yet significant, increase was observed in albumin level only in saline treated groups, after 24 hours. The increased serum albumin value (2.6 mg/dL) still fell within the normal range for albumin levels in rats (Ringler and Dabich, 1979).

5b. Hematological Profile in Rats after Administration of rHV2

The variations in hematological parameters were evaluated after i.v. and s.c. administration of rHV2 in rats. Hematological markers such as cell counts, hemoglobin content and related parameters were measured. The hematology profile in rats used in this study (Tables 15 and 16) indicated normal baseline values for all markers, as reported in previous studies (Ringler and Dabich, 1979; Lang, 1993a).

I.V. Administration. There was no significant variation in white blood cell (WBC) count after rHV2 treatment, when compared to saline treated control rats (Table 15). The significant decreases in red blood cell (RBC) number, hemoglobin (HGB) and hematocrit (HCT) observed in both the saline and rHV2 treated groups may be due to blood loss during repeated blood sampling. However, in both treatment groups, erythrocyte indices such as mean cell volume (MCV), mean cell hemoglobin (MCH) and mean cell hemoglobin concentration (MCHC) did not vary from corresponding baseline values, at 24 hours. This rules out the presence of any microcytic or macrocytic anemia (Nelson and Morris, 1984), as the above parameters are calculated from corresponding RBC count, HGB and HCT values.

The platelet count was reduced moderately (significant, $p<0.05$) in rHV2 treated rats, compared to baseline and saline treated rats. This reduction in platelet count may not be critically thrombocytopenic as the

platelet count still remained within the reported normal range for rats (Lang, 1993a). In addition, there was no variation in the mean platelet volume (MPV) after rHV2 administration. Nonetheless, this observation of moderately reduced platelet count needs to studied more extensively in different experimental settings.

S.C. Administration. There was no change in WBC count after s.c. rHV2 administration (Table 16), as seen in the i.v. studies. Again, there were significant reductions in RBC count, HGB and HCT after rHV2 administration, compared to corresponding baseline values. This finding can be explained by the blood loss due to repeated sampling procedure as there was a decrease in HCT in saline treated groups, as well (Table 16). Once again, no variations were observed in erythrocyte indices such as MCV, MCH and MCHC.

The platelet count was normal and unchanged after s.c. administration of rHV2, though there was a trend toward a slight, but insignificant, decrease in thrombocytes after rHV2 administration (Table 16). More studies are required to rule out the possibility of any thrombocytopenia induced by recombinant hirudin, particularly after long term therapy.

It may be concluded that none of the observed alterations in serum chemistry and hematological parameters after single dose administration in rats, may be considered drug-related.

6. Investigation of Pharmacokinetics and Pharmacodynamics of rHV2 after Single Dose Administration in Dogs

The purpose of this set of experiments was to evaluate the pharmacokinetics and pharmacodynamics of rHV2 after increasing single dose administration in dogs. These studies were similar to the experiments in rats, but also included a series of *ex vivo* coagulation results. Both intravenous and subcutaneous routes were used, as before. The results from the single dose studies in dogs enabled the selection of an appropriate dose for the multiple dose study in dogs, to be discussed in the following section.

I.V. Administration. The pharmacokinetic analysis of i.v. administration indicated that rHV2 exhibits linear pharmacokinetics, in agreement with the results obtained in rats. The $t_{1/2}$, V_d and Cl_{tot} values did not vary between the three increasing doses of rHV2 (Table 17). At the same time, the $AUC_{0-\infty}$ and C_{max} values increased dose dependently. The V_d values of 200 to 250 mL/kg indicated that there was an extravascular component in the distribution of rHV2 in dogs.

The time course of *ex vivo* coagulation tests was very similar to that obtained in the time course studies in rabbits. The progression of APTT (OT) (Figure 55) and APTT (Dade) (Figure 56) was comparable to the pharmacokinetic curve of rHV2 after single dose, i.v. administration. Dose dependent peak increases were obtained in both APTT (OT) and APTT (Dade). However, in the case of APTT (Dade), no distinction was observed in the

decline of responses between the three doses, after the peak response.

The time course of *ex vivo* antithrombin activity was also evaluated using the thrombin based coagulation tests: TT (20 U/mL), Ca^{+2}TT (10 U/mL) and Ca^{+2}TT (20 U/mL), as depicted in Figures 57, 58 and 59, respectively. The TT (20 U/mL) pointed toward the fact that rHV2 was a highly potent inhibitor of thrombin, leading to antithrombin responses that are beyond the sensitivity range (>300 seconds), at very low plasma rHV2 concentrations. As the TT (20 U/mL) values started declining after 30 minutes, the dose dependent antithrombin effect of rHV2 became more clear (Figure 57).

Ca^{+2}TT (10 U/mL and 20 U/mL) tests were performed to obtain more definite end points at high concentrations of rHV2 in plasma. However, coagulation responses remained steady at >300 seconds using both of these tests, up to at least 15 to 30 minutes after rHV2 administration, in escalating i.v. doses. Again, as the clotting responses using Ca^{+2}TT (10 and 20 U/mL) declined, the time response curves were oriented in a dose dependent fashion. An attempt was made to evaluate the antithrombin activity of rHV2 after increasing the calcium-thrombin content in the reagent to obtain Ca^{+2}TT (30 U/mL). However, the baseline value obtained for this test was only 3 to 4 seconds. Plasma samples from time intervals after varying circulation times of rHV2 exhibited no measurable increase beyond the baseline values. These results indicated that a delicate balance is required to be maintained in the composition of the calcium-thrombin reagent, in order to obtain clotting

responses within the sensitivity range of the assay using the Fibrometer®.

The anti-IIa assay, using the chromogenic thrombin substrate method, appeared to be the most reliable *ex vivo* method to predict antithrombin activity of rHV2. Thrombin inhibitory responses increased linearly with increasing i.v. doses of rHV2. The progression of anti-IIa responses matched the time course of plasma concentrations of rHV2 after i.v. administration in dogs.

S.C. Administration. The pharmacokinetics of rHV2 after s.c. administration indicated that rHV2 exhibits linear pharmacokinetics, similar to the i.v. results. This was evident from the dose dependent increases in C_{max} and $AUC_{0-\infty}$ values (Table 18). Also, there were no significant differences between the three doses in V_d, $t_{1/2}$, and Cl_{tot} rates.

The average bioavailability of rHV2 after s.c. administration was almost equal to 98%, when compared to that after i.v. administration. However, the C_{max} values obtained after each s.c. dose were considerably lower than obtained with corresponding i.v. doses (Figure 54, i.v. and Figure 61, s.c.). A lower antithrombin activity was also observed at the peak rHV2 levels after each s.c. administration (Figure 60, i.v. and Figure 67, s.c.). The relative F of 98% after s.c. administration may be due to the sustained levels of rHV2 in plasma for an extended period of time (1 to 4 hours). In addition, it may be possible that the rHV2 molecule is modified after s.c. administration, to

produce a lower functional activity (anti-IIa assay, Figure 67), whilst retaining its immunological activity in the ELISA method. This finding agrees with previous results with single dose i.v. and s.c. administration of rHV2 (Iyer *et al.*, 1993a).

The time course study of *ex vivo* antithrombin activity of rHV2 after s.c. administration exhibited considerable variability in the responses between various dogs. The reason for this finding may be due to the possible differences in the subcutaneous barrier between dogs.

It was evident that the bioavailability after s.c. administration varied between rats, rabbits and dogs. It may be speculated that this may be due to differential metabolic pathways and degradation patterns of rHV2 between the three species. Differential bioavailability patterns may be observed due to variations in enzymatic degradation and thickness of cutaneous barrier. rH has been shown to exhibit significant extra-renal clearance in rats (Appendix I). The exact mechanism of rHV2 metabolism in rats is not known.

There was no definite distinction in the time course of APTT (OT) after the higher two doses (0.5 and 1.0 mg/kg, s.c.) of rHV2 (Figure 62). In the case of APTT (Dade), there was no difference between the three doses in the absorption phase, though the decline of responses after the peak appeared to be dose dependent (Figure 63).

The thrombin based coagulation tests were performed in the s.c. studies, in an effort to obtain dose dependent dose-response curves.

Evaluation of *ex vivo* antithrombin effects of rHV2 using TT (20 U/mL) exhibited a sustained maximum response between about 60 and 180 minutes after s.c. administration of the higher two doses (Figure 64). A sharp peak was obtained in $Ca^{+2}TT$ (10 U/mL) and $Ca^{+2}TT$ (20 U/mL) responses, after administration of 0.5 and 1.0 mg/kg of rHV2, as shown in Figures 65 and 66, respectively. These results indicated that more definite end points in responses could be obtained using calcium-thrombin reagents to monitor *ex vivo* anticoagulant effects of rHV2.

The time course of amidolytic anti-IIa activity exhibited distinct dose dependent increases in thrombin inhibitory activity of rHV2 (Figure 67). It was interesting to note that the peak anti-IIa response was only about 50%, after administration of the highest s.c. dose, i.e. 1.0 mg/kg, of rHV2. This may be due to the decreased availability of rHV2 after s.c. administration, or possible chemical modifications in rHV2 after s.c. administration, resulting in functional modifications of this agent.

7. Investigation of Pharmacodynamics of rHV2 after Repeated Administration in Dogs

The purpose of repeated administration of rHV2 was to determine any possible accumulation of rHV2 in dogs with time. This could lead to an increased sensitivity in antithrombotic activity of rHV2, and possibly to untoward side effects such as bleeding. On the other hand, repeated rHV2 administration could lead to formation of neutralizing antibodies to rHV2,

resulting in decreased intensity of functional responses, and hence, a compromised therapeutic efficacy.

I.V. Administration. Measurement of plasma concentrations of rHV2 at time periods of 6, 30 and 60 minutes, after each dose, showed that there was no significant accumulation or loss of rHV2 from plasma (Figure 68). No major alterations in the availability or disposition of the drug was noted with repeated i.v. administration (Table 19). Similarly, no major changes were observed in the *ex vivo* analysis tests, such as APTT (Dade), $Ca^{+2}TT$ (10 U/mL) and anti-IIa assay (Figures 69 to 71, respectively).

S.C. Administration. Similar to the i.v. results, there was no indication of possible accumulation or inactivation of rHV2 in plasma after repeated, daily, s.c. administration of rHV2, with respect to individual plasma levels (Figure 72). However, there was a trend toward a slight accumulation of rHV2, as indicated by the increase in AUC values and decrease in clearance values (Table 19). This finding may be due to the probable conversion of rHV2 to inactive metabolites, which may retain their immunogenicity. This argument is substantiated by the fact that there was no major variation in the *ex vivo* responses, as shown by the time course of APTT (Dade), $Ca^{+2}TT$ (10 U/mL), and anti-IIa assay (Figures 73 to 75, respectively).

No visible signs of excessive bleeding were evident at injection sites and blood sampling sites on the dogs used in the study, after each dose of rHV2.

Previous studies with repeated administration of rHV2 (2 mg/kg, i.v.) at a dosing interval of one week (for three weeks), pointed toward possible accumulation of rHV2 in plasma, and a trend toward sensitization of *ex vivo* antithrombin responses (Iyer *et al.*, 1993b). However, the dose used was twice the current dose, and the dosing regimen was different. Bichler *et al.* (1991) observed no formation of hirudin specific antibodies after repeated i.v. administration of natural and recombinant hirudin in baboons.

The results from these studies indicated that recombinant hirudin may be used safely at daily i.v. and s.c. injections up to a period of one week without any significant accumulation of rHV2, or sensitization or desensitization of *ex vivo* antithrombin responses. The experiments were carried out in apparently healthy dogs, with normal renal function. It has been shown that plasma rH levels remained constant up to 120 minutes after i.v. administration in nephrectomized dogs (Nowak *et al.*, 1988). Hence, it is to be emphasized that a renal function compromise due to age, drug or disease, may significantly alter the results. Therefore, individual studies are needed in the stated groups, as the study outcome is dependent on several factors.

7a. Serum Chemistry Profile in Dogs after Repeated rHV2 Administration

The purpose of this study was to investigate possible changes in physiological function in dogs after chronic administration for one week.

I.V. Administration. The serum chemistry markers at baseline agreed with reported normal values on some parameters in dogs (Schalm *et al.*, 1975) and routine profiles performed in Hemostasis Research Laboratories. There were no fluctuations in liver enzymes at a daily i.v. dose of 1 mg/kg of rHV2, as indicated by the normal levels of ALP, AST and ALT (Figure 78) when compared to baseline, prior to initial dose, using the two-way repeated measures ANOVA. The significant ($p<0.1$) day-to-day variation in γ-GT values (Figure 79) may not be of major concern, as the values returned to normal levels, by the sixth day. Serum electrolytes (Figure 77), as well as BUN and creatinine (Figure 78) did not vary with repeated rHV2 administration, indicating normal renal function. The observed normal levels of total protein, total bilirubin and albumin indicated normal protein synthesis (Figure 76 and 79). Normal phosphorous and calcium homeostasis were also evident (Figures 76 and 77, respectively).

The significant ($p<0.1$) day-to-day fluctuations in total CO_2 may be due to the stress of daily rHV2 administration and repeated blood sampling. Conscious dogs were used; hence, any ventilation/ perfusion disorders due to anesthetics may be ruled out. There were normal levels of LDH, triglycerides, glucose and cholesterol throughout the period of repeated rHV2 administration (Figure 80).

S.C. Administration. Normal electrolyte and lipid levels, renal function, protein synthesis, calcium and phosphorous homeostasis and functional hepatic integrity, and a non-diabetic state were observed after repeated s.c. administration of rHV2, at a dose of 1 mg/kg (Figures 81 to 85). The significant step-wise decrease in LDH levels after rHV2 administration on most days may be due to the stress induced by repeated blood sampling. Nonetheless, this observation requires further clarifications.

7b. Hematological Profile after Repeated rHV2 Administration in Dogs

I.V. Administration. The hematological profile after repeated i.v. administration of rHV2 indicated that there were no major variations in most parameters (Figures 86 to 88). However, by the sixth day of the study, there was almost a 13% decrease in HCT, and a 20% decrease in HGB levels. This finding may be due to the repeated blood draws for an extended period of time.

S.C. Administration. There was a significant variation in HCT and HGB levels after repeated rHV2 administration, in agreement with the results from the i.v. study. Most other parameters exhibited negligible variations with time (Figures 89 to 91).

In conclusion, it may be stated that the observed alterations in some of the serum chemistry and hematological parameters after multiple rHV2 administration in dogs, may not be drug-related.

CHAPTER VI

SUMMARY

This dissertation addressed the pharmacokinetic and pharmacodynamic investigation of rHV2, with particular reference to the utilization of existing global tests for the monitoring of the anticoagulant effects. In addition, thrombin inhibition assays and absolute quantitation of rHV2 were accomplished by developing optimized methods. From the pharmacodynamic studies, additional information on the relative therapeutic safety and potential toxicity of rHV2 was obtained. A summary of results from the major experimental protocols included in this study, is listed below:

In Vitro Studies

1. Species and assay dependent anticoagulant effects of rHV2 were observed, utilizing the existing APTT reagents, modified TT assays and an amidolytic anti-IIa assay. These methods were capable of detecting hirudin in original concentration ranges of 0.01 to 5 μg/mL. However, the linear ranges varied markedly. In the amidolytic anti-IIa assay, while the rabbit, rat and dog plasma provided similar results, the rat plasma required much higher (10-fold) concentrations to exhibit linear inhibitory responses.

2. This dissertation provided valid data on thrombin titration method as a means to evaluate the potency of rHV2 and related antithrombin agents. rHV2 was found to exhibit a specific activity of ≈ 15,873 ATU/mg (1.1×10^8 ATU/mM).

3. A polyclonal antibody based competitive ELISA method provided a procedure to measure the absolute concentrations of rHV2 in plasma obtained from different species. A concentration dependence on the immunoreactivity of rHV2 enabled the quantitation of rHV2 in various plasma systems for the determination of pharmacokinetic parameters. The linear range of this assay was between 25 and 1000 ng/mL.

In Vivo Studies

Antithrombotic and Hemorrhagic Profile of rHV2

1. rHV2 produced a dose dependent antithrombotic effect in a modified rabbit jugular vein stasis thrombosis model. The apparent ED_{50} after i.v. administration of rHV2 was in the range of 9-11 μg/kg, at a time period of 5 minutes. The apparent ED_{50} after s.c. administration of rHV2 was 90-220 μg/kg, at 2 hours.

2. rHV2 also produced a dose dependent increase in the blood loss, as measured in a modified ear blood loss model in rabbits. The apparent TD_{50}

values of rHV2 after i.v. and s.c. administration were 0.7-0.8 mg/kg, and 0.8-2.8 mg/kg, respectively. No significant blood loss was measured after administration of experimentally determined therapeutic doses of rHV2, when compared to saline treated control rabbits.

3. The protective index, as calculated from antithrombotic and bleeding responses at circulation times of rHV2 corresponding to observed peak plasma rHV2 levels in rabbits, was found to be 78 (i.v.) and 9 (s.c.)., indicating a wide margin of safety.

Pharmacokinetic Profile of rHV2

1. The pharmacokinetic parameters in all three species were calculated from the absolute levels (ELISA method) using non-compartmental methods. In rabbits, the terminal elimination half-life ($t_{1/2}$) was 55 and 24 minutes after i.v. and s.c. dosing of rHV2, respectively. The results from this study were consistent with previously reported data. The biological residence time after s.c administration was much higher after s.c. administration (mean residence time/MRT of $>$ 3 hours after s.c. administration). The V_d obtained from the i.v. studies demonstrated that there was an extravascular component in the distribution of rHV2 in rabbits. The relative bioavailability (F) after s.c. administration was 45%, when compared with i.v. administration.

2. The pharmacokinetics of rHV2 was studied in rats after escalating i.v. (0.1, 0.4 and 0.5 mg/kg) and s.c. (0.1, 0.5 and 1.0 mg/kg) administration.

The mean $t_{1/2}$ was $\approx$ 22.2 and 40 minutes, after i.v. and s.c. administration, respectively. No differences in $t_{1/2}$, time to reach maximum concentration (t_{max}), clearance rate (Cl_{tot}) and volume of distribution (V_d) values were noted after step-wise dose increment in both i.v. and s.c. studies. However, a dose dependent increase in area under the plasma concentration-time curves ($AUC_{0-\infty}$) and maximum concentrations (C_{max}) values was apparent. These results indicated that rHV2 exhibited linear pharmacokinetics in rats. The V_d values indicated extravascular distribution, as observed in rabbits.

3. The pharmacokinetics of rHV2 was also studied in a dog model after increasing i.v. (0.25, 0.5 and 1.0 mg/kg) and s.c. (0.25, 0.5 and 1.0 mg/kg) doses. The mean $t_{1/2}$ was $\approx$ 60 minutes after i.v. and s.c. administration in dogs, consistent with previous results. rHV2 exhibited linear pharmacokinetics in the dog model, as well. This was evident from dose dependent increases in C_{max} and $AUC_{0-\infty}$, and no major variations in $t_{1/2}$, V_d and Cl_{tot} parameters between the three i.v. and s.c. doses. Extravascular distribution was also observed in dogs, similar to the results from the rat and rabbit studies. The F value was $\approx$ 98% after s.c. administration of rHV2 in dogs, indicating species differences in absorption, distribution and degradation mechanisms of rHV2.

4. Utilizing the single dose ^{3}H-inulin clearance method, no effect on renal function was observed when compared with saline treated rats. rHV2 was used in doses of 0.5 mg/kg, i.v. and 1 mg/kg, s.c. Thus, rHV2, by itself,

did not produce any renal compromise in rats.

Pharmacodynamic Profile of rHV2

1. In the dose-ranging study in rabbits, rHV2 exhibited dose and route of administration dependent *ex vivo* anticoagulant and anti-IIa activity. rHV2 also exhibited a time dependent *ex vivo* anticoagulant and anti-IIa activity in rabbits, as observed in the time-course experiments.

2. The *in vivo* antithrombotic activity of rHV2 was found to be time dependent, after the administration of a dose of 25 μg/kg (i.v.) and 375 (s.c.). In the i.v. studies, the apparent $t_{1/2}$ of antithrombotic effect at 10 minute stasis, was greater than 75 minutes, whereas after the 20 minute stasis time, the $t_{1/2}$ was approximately 10 minutes. In the s.c. studies, persistent, complete antithrombotic response lasted for up to 6 hours, at the 10 minute stasis time. In the 20 minute stasis study, complete inhibition of the thrombus formation was noted at 2 hours, after which time, there was a gradual loss of antithrombotic activity, reverting to baseline level at 4 hours.

3. The time course of *ex vivo* antithrombin activity of rHV2 was also studied in rats, using the amidolytic anti-IIa assay. There were dose dependent increases and time and route dependent variations in anti-IIa activity of rHV2 in this study.

4. Dose, time and route dependent *ex vivo* anticoagulant/ antithrombin activity was also observed after increasing i.v. and s.c.

administration of rHV2 in dogs.

Integrated Pharmacokinetics and Pharmacodynamics of rHV2

1. Significant correlations were obtained between plasma concentrations of rHV2 determined from the ELISA method, and antithrombotic as well as antithrombin/ anticoagulant activity of rHV2. This finding depended on the species, route of administration and individual assay method used.

2. The concentration-effect relationship after i.v. administration of rHV2 in rabbits could be described by the sigmoidal E_{max} model. This indicated a direct relationship between plasma rHV2 concentrations and observed *in vivo* antithrombotic responses. The model can be used to predict magnitudes of responses in future.

3. To simulate the prophylactic use of rHV2, a repeated dosage study was performed in dogs (1 mg/kg, i.v. and s.c. for one week). In the anticoagulant assays, no evidence of accumulation or loss of this agent was evident. However, in the ELISA method, an initial phase of equilibration, resulting in a progressive increase in circulating rHV2 levels was noted for a period of up to two days after i.v. administration. After this, a uniform concentration profile was evident. In contrast, in the s.c. studies, all assays revealed a uniform concentration and antithrombin profile after all doses.

Clinical Laboratory Profile after rHV2 Administration

1. The *ex vivo* analysis of the sera samples from rats treated with 0.5 mg/kg (i.v.) and 1 mg/kg (s.c.) did not reveal any alteration of hematological parameters, when compared to saline treated rats. All parameters were comparable in the clinical chemistry profile, with the exception of a decrease in triglycerides and lactic dehydrogenase (LDH). These results indicate that rHV2 administration, at these doses and route, does not produce any toxic responses, as measured by clinical laboratory profile.

2. Clinical laboratory profile was also studied after repeated rHV2 administration in dogs (1 mg/kg, i.v. and s.c., for one week). The *ex vivo* analysis of sera obtained from the repeated i.v. and s.c. studies did not reveal any major alterations in hematological and clinical profile. However, a suggestive day-to-day variation in total CO_2 (i.v.) and LDH (s.c.) was noted, however. Consistent with rat studies, these data, once again, indicate the non-toxic nature of rHV2 at the given dosage schedule in dogs.

CHAPTER VII

CONCLUSIONS

1. Thrombin titration and polyclonal antibody based ELISA methods have been developed to determine functional potency and absolute concentrations of recombinant hirudin variant 2 (rHV2), respectively.
2. While species dependent variations in the anticoagulant and anti-IIa responses with rHV2 were obvious, these methods can be used to monitor the antithrombin effects of this drug.
3. rHV2 represents a drug that exhibits a wide margin between antithrombotic and hemorrhagic responses after i.v. administration: broad therapeutic window.
4. rHV2 did not compromise renal function, or produce significant any alterations in clinical laboratory profile.
5. rHV2 exhibited linear pharmacokinetics in two animal species, i.e. rats and dogs, and demonstrated extravascular distribution in all three species.
6. This agent may be used safely in repeated i.v. administration protocols.
7. The time course studies indicated a direct correlation between pharmacokinetics and pharmacodynamics of rHV2.

8. The *in vivo* pharmacodynamic/pharmacokinetic relationship of rHV2 may be described using a sigmoidal E_{max} model, after i.v. administration in rabbits.

9. These studies demonstrated that rHV2 is a potent anticoagulant and antithrombotic agent with a predictable pharmacokinetic/ pharmacodynamic relationship, and a desirable therapeutic index.

APPENDIX I

PHARMACOKINETIC SURVEY OF NATURAL AND RECOMBINANT HIRUDIN

TABLE 20

PHARMACOKINETIC SURVEY ON NATURAL AND RECOMBINANT HIRUDIN (PART I)

Reference	Hirudin	Subjects	Dose & Route	Assay	Results
Markwardt *et al.*, 1982	Natural	Rats Rabbits Dogs	10,000 ATU/kg, i.v. bolus. 200 ATU/kg, infusion (rats)	Chromogenic Substrate Method	$t_{1/2\ (\alpha)} = 10$ min. $t_{1/2\ (\beta)} =$ 51 min. (dogs);62 min.(rabbits);56 min.(rats) 70% of hirudin administered was found in urine within the first hour.
Markwardt *et al.*, 1984	Natural	Humans (male)	1000 ATU/kg, i.v. bolus, s.c.	Chromogenic Substrate Method	<u>i.v.bolus</u> $t_{1/2(\alpha)} = 0.15$ hr. $t_{1/2(\beta)} = 0.84$ hr. $V_{d(ss)} = 12.9$ L AUC = 5.69 ATU.hr./mL $Cl_{tot} = 230$ mL/min. $Cl_{ren} = 99.5$ mL/min. 45% of hirudin administered was recovered in urine <u>s.c.</u> $t_{1/2(\beta)} = 0.64$ hr. $k_a = 1.1$ 1/hr. F = 36% 30% of administered hirudin recovered in urine

TABLE 21

PHARMACOKINETIC SURVEY ON NATURAL AND RECOMBINANT HIRUDIN (PART II)

Reference	Hirudin	Subjects	Dose & Route	Assay	Results
Richter *et al.*, 1988	Natural; ^{125}-I labelled	Dog Rat	0.5 mg/kg (dog) 1 mg/kg (rat) i.v., s.c., i.v. infusion	Thrombin Binding Assay (microcolumns of thrombin-sepharose)	**Rat** i.v.: $t_{1/2(\alpha)}$ = 12.4 min.; $t_{1/2(\beta)}$ = 65 min.; $V_{d(ss)}$ = 0.22 L; $AUC_{0\text{-}5h}$ = 1.74 μg.hr/mL; Cl_{tot} = 170 mL/min infusion: $t_{1/2}$ = 2 hr. %recovery: 15.1%-i.v.; 11.0%-s.c.; 14.8%-inf. **Dog** i.v.: $t_{1/2(\alpha)}$ = 12.5 min.; $t_{1/2(\beta)}$ = 56.6 min.; $V_{d(ss)}$ = 4.55 L; $AUC_{0\text{-}5h}$ = 1.38 μg.hr/mL; Cl_{tot} = 7060 mL/min. infusion: $t_{1/2(\beta)}$ = 5 hr. %recovery: 87.6%-i.v.; 58.7%-s.c.; 67.6%-inf.
Bichler *et al.*, 1988	Natural	Humans (3 females 9 males)	600, 800, 1000 ATU/kg,s.c. 1000 ATU/kg, i.v.	Radioimmuno bioassay (RIBA)	s.c.: $t_{1/2(\alpha)}$ = 74.8 min.; $t_{1/2(\beta)}$ = 101.3 min.; Cl_{tot} = 153 mL/min.; Cl_{ren} = 92.6 mL/min.; CUE = 61.2% i.v.: $t_{1/2(\alpha)}$ = 9.2 min.; $t_{1/2(\beta)}$ = 64.7 min.; Cl_{tot} = 187.1 mL/min.; Cl_{ren} = 79.9 mL/min.; AUC = 0.677 μg.hr/mL; $V_{d(ss)}$ = 0.24 L/kg; CUE = 42.5%

TABLE 22

PHARMACOKINETIC SURVEY ON NATURAL AND RECOMBINANT HIRUDIN (PART III)

Reference	Hirudin	Subjects	Dose & Route	Assay	Results
Markwardt *et al.*, 1988a	Recombinant; (*E.Coli*, Genbiotec) ^{125}I-labelled	Rats Dogs	1 mg/kg, i.v., s.c., i.v. infusion, intratracheal instillation rectal	Chromogenic Substrate Assay % radioactivity measured in urine	**Rats** i.v. $t_{1/2(\alpha)} = 0.15$ hr. $t_{1/2(\beta)} = 1.07$ hr. $AUC_{0-5h} = 1.64$ µg.hr/mL $V_{d(ss)} = 0.66$ L/kg $V_c = 0.31$ L/kg $Cl_{tot} = 3.05$ mL/min. s.c. $k_a = 2.3$ 1/hr. $t_{1/2(\beta)} = 1.9$ hr Instillation $t_{1/2(\beta)} = 200$ min. Urinary excretion i.v.-15.1%, inf.-14.8%, s.c.-11%, inst.-1.8% *No marked absorption after rectal administration* **Dogs** i.v. $t_{1/2(\alpha)} = 0.25$ hr. $t_{1/2(\alpha)} = 1.22$ hr $AUC_{0-6h} = 1.54$ µg.hr/mL $V_{d(ss)} = 0.28$ L/kg $Cl_{tot} = 183$ mL/min $Cl_{ren} = 152$mL/min s.c. $t_{1/2(\beta)} = 5.75$ hr. 70% recovered in urine in active form
Markwardt *et al.*, 1988b	Recombinant (*S.cerevisiaeae* Hoechst AG)	Humans (females)	0.1 mg/kg, i.v. bolus, i.m., s.c.	Chromogenic Substrate Method	i.v. bolus $t_{1/2(\alpha)} = 0.15$ hr. $t_{1/2(\beta)} = 0.91$ hr. $AUC_{0-6} = 0.54$ µg.hr/mL $V_{d(ss)} = 8.9$ L $Cl_{tot} = 168$ mL/min. $Cl_{ren} = 58.2$ mL/min. 34.6% recovery in urine. s.c. $k_a = 1.1$ 1/hr. 28.5% recovery in urine. i.m. $k_a = 1$ 1/hr. 40.7% recovery in urine.

TABLE 23

PHARMACOKINETIC SURVEY ON NATURAL AND RECOMBINANT HIRUDIN (PART IV)

Reference	Hirudin	Subjects	Dose & Route	Assay	Results
Nowak *et al.*, 1988	Recombinant (*E.Coli*, Genbiotec)	Dogs, normal, nephrectomized	0.5 mg/kg, i.v., s.c., i.v. infusion	Chromogenic Substrate Assay	i.v. 70% excreted within 1 hr. Plasma rH levels remained constant after 120 min. in nephrectomized dogs More pronounced renal clearance of rH than natural hirudin s.c. $t_{1/2(\beta)}$ = 5.75 hr. AUC_{0-6} = 1.71 μg.hr/mL
Markwardt *et al.*, 1989	Recombinant (CGP 39393, Ciba) rHV2	Rats	1 mg/kg 2 mg/kg i.v. bolus	Thrombin Clotting time	$t_{1/2(\alpha)}$ = 0.08 hr. $t_{1/2(\beta)}$ = 1.18 hr. $V_{d(ss)}$ = 0.63 L/kg AUC_{0-3h} = 3.81 μg.hr/mL Cl_{tot} = 2.09 mL/min.
Meyer *et al.*, 1990	Recombinant (Hoechst AG)	Humans (males)	0.01, 0.02, 0.04, 0.07, 0.1 mg/kg; i.v. bolus	Chromogenic Substrate Assay	*Existence of third compartment cannot be excluded* AUC and C_{max} - dose related, Cl and $V_{d(ss)}$ - constant with increasing doses, total urinary excretion proportional to increasing doses - *Linear Pharmacokinetics* 0.1 mg/kg $t_{1/2(\alpha)}$ = 0.1 hr. $t_{1/2(\beta)}$ = 0.82 hr. $V_{d(ss)}$ = 14.3 L. Cl_{tot} = 205 mL/min. Cl_{ren} = 94.2 mL/min.

TABLE 24

PHARMACOKINETIC SURVEY ON NATURAL AND RECOMBINANT HIRUDIN (PART V)

Reference	Hirudin	Subjects	Dose & Route	Assay	Results
Markwardt *et al.*, 1991	Dextran bound recombinant hirudin (Hoechst AG)	Rabbits Rats	10000 ATU/kg (rats, rabbits), 10,00 ATU/kg (rats)	Thrombin Inhibition Assay	Dextran 40-hirudin: Rats **1000 ATU/kg:** $t_{1/2(\alpha)}$ = 0.28hr. $t_{1/2(\beta)}$ = 6.3 hr. V_d = 36.1 mL AUC_{0-6h} = 50.2 ATU.hr/mL **10,000 ATU/kg:** $t_{1/2(\alpha)}$ = 0.2 hr. $t_{1/2(\beta)}$ = 6.9 hr. V_d = 36.6 mL AUC_{0-6h} = 370 ATU.hr/mL Rabbits **1000 ATU/kg:** $t_{1/2(\alpha)}$ = 0.4hr. $t_{1/2(\beta)}$ = 6.3 hr. V_d = 336mL AUC_{0-6h} = 46 ATU.hr/mL Similar results with dextran 70-hirudin

TABLE 25

PHARMACOKINETIC SURVEY ON NATURAL AND RECOMBINANT HIRUDIN (PART VI)

Reference	Hirudin	Subjects	Dose & Route	Assay	Results
Bichler *et al.*, 1991	Natural and recombinant hirudin (plantorgan)	Baboons	1000 ATU/kg on days 1, 3, 8 and 42	Indirect ELISA Competitive ELISA for detection of antibodies. Radioimmunobioassay for measurement of hirudin (Direct Skin test and *in vitro* histamine release)	No evidence of immune response to natural and recombinant hirudin - poor immunogenic potential. 27 % of administered dose recovered in urine. Half-life of urinary elimination was 27 minutes (shorter than humans - 120 min.)
Bichler *et al.*, 1993	Recombinant hirudin, dilactitol ^{125}I-tyramine (*I-DLT)-labelled	Rats	20-60 µg/kg for hirudin, 5-20 µg/kg for hirudin-thrombin complex, 5 µg/kg for thrombin-AT complex	Chromogenic substrate assay Measurement of radioactivity in tissues	Majority of radioactivity recovered in kidney for all three labelled entities. Conjugation of *I-DLT to hirudin did not influence its pharmacokinetic behavior. Hirudin is catabolized in kidney; thrombin-hirudin complex is catabolized in liver and kidney.

APPENDIX II

SPECIFICATIONS ON rHV2
(BATCH RHE 15, Sanofi Recherche, France)

SANOFI RECHERCHE - CENTRE DE TOULOUSE
195, ROUTE D'ESPAGNE
B.P. 1169 - 31036 TOULOUSE CEDEX (FRANCE)
TEL.: 61 43 22 00
TELEX: 531 535
TELECOPIE: 61 43 22 01

sanofi RECHERCHE

Ms Lalitha Iyer
Dept. of Pharmacology
Loyola University
2160 South First Avenue
MAYWOOD
Illinois 60656
U.S.A.

Our ref. : JPM/AJP/130/91 10th October 1991

Dear Ms Iyer,

In response to the questions you asked in your letter, you will find hereafter some answers and articles which may be of use to you.

1. The compound in solution is stable for 24h at 25°C and for 7 days at 5°C. Concerning the second part of your question, a thorough study has not been carried out but we think that hirudin which has been frozen and thawed once can be used without any problem. It is however advisable not to freeze and thaw several times.

2. RHE15 vials contain 10.9 mg of hirudin. The specific activity is 14495 ATU/mg.

3. The complete amino acid sequence is shown in the enclosed article : *The structure of a complex of recombinant hirudin and human a-thrombin,* RYDEL TJ et al.

4. Yeast expression vector used : plasmid is an E.coli yeast shuttle vector designed to direct the secretion of foreign proteins in yeast. It contains the 2-µm segment and the yeast LEU2 gene as well as the Ap^R gene and the origins of replication of pBR322.

5. Molecular weight is 6906 as determined by MS analysis.

6. Plasma concentration determination by the following methods : (see attached sheets)
   - thrombin time (TT)
   - activated cephalin time (TCA)
   - chromogenous substrate

Urine concentration determination by chromogenous substrate assay.

7. We do not have any radiolabelled hirudin available. See attached article : *Site specific radioiodination of recombinant hirudin,* TUONG et al.

Trusting that this information will help you in your doctoral dissertation,

Yours Sincerely

Jean-Pierre MAFFRAND
Scientific Director
Toulouse Research Centre

Encl.

cc : J. FAREED, J.M. HERBERT

SANOFI RECHERCHE - CENTRE DE TOULOUSE
195, ROUTE D'ESPAGNE
B.P. 1169 - 31036 TOULOUSE CEDEX
TEL.: 61 43 22 00
TELEX: 531 535
TELECOPIE: 61 43 22 01

sanofi RECHERCHE

CERTIFICATE OF ANALYSIS

- Analysis n° : 2775
- Date of the request : 26-Nov-1990
- Page : 1/3

SR 29010
10 mg SR 29010 POWDER FOR PARENTERAL USE

- Supplier : SANOFI RECHERCHE MONTPELLIER
- Reference : 1A1
- Batch : RHE15
- Batch size: 782 units
- Quantity : 581 Units
- Storage conditions : + 4° C
- Date of receipt : 26-Nov-1990
- Manufacture date : 15-May-1990
- Expiration date : 15-May-1991
- Number of containers : 1

- Monograph : CCR4416
- Edition : 1

TESTS	SPECIFICATIONS	RESULTS
CHARACTERS		
Appearance	Complies	Complies (1)
IDENTIFICATION		
Mannitol	Complies	Complies (1)
Active ingredient		
- Reverse phase chromatography	Complies	Complies
- Anionic exchange chromatography	Complies	Complies
- Exclusion chromatography	Complies	Complies
TESTS		
Uniformity of mass	Complies	Complies (1)
Reconstituted solution appearence	Not more opalescent than reference suspension II, colorless	Not more opalescent than reference suspension II, colorless
pH of the reconstituted solution	3.5 to 5.5	5.43

SANOFI RECHERCHE - CENTRE DE TOULOUSE
195, ROUTE D'ESPAGNE
B.P. 1169 - 31036 TOULOUSE CEDEX
TEL.: 61 43 22 00
TELEX: 531 535
TELECOPIE: 61 43 22 01

sanofi RECHERCHE

CERTIFICATE OF ANALYSIS (cont'd)

SR 29010
10 mg SR 29010 POWDER FOR PARENTERAL USE
Analysis n° : 2775 Date of the request : 26-Nov-1990
Batch : RHE15 Page : 2/3

TESTS	SPECIFICATIONS	RESULTS
Water content	not more than 3.0 per cent	1.4 per cent (1)
Impurities by reverse phase chromatography	not more than 5.0 per cent	2.4 per cent
Impurities by anionic exchange chromatography	not more than 5.0 per cent	0.6 per cent
Impurities by exclusion chromatography	not more than 3.0 per cent	0.8 per cent
Sterility	Complies	Complies (1)
Pyrogens	Complies	Complies (1)
Abnormal toxicity	Complies	not performed
Tightness	Complies	not performed
ASSAY		
Reverse phase chromatography (on the substance as it is, per vial)	9.0 to 11.0 mg	11.18 mg

OBSERVATIONS

(1) Results of the initial control .

SANOFI RECHERCHE - CENTRE DE TOULOUSE
195, ROUTE D'ESPAGNE
B.P. 1169 - 31036 TOULOUSE CEDEX
TEL.: 61 43 22 00
TELEX: 531 535
TELECOPIE: 61 43 22 01

sanofi RECHERCHE

CERTIFICATE OF ANALYSIS (cont'd)

SR 29010
10 mg SR 29010 POWDER FOR PARENTERAL USE
Analysis n° : 2775 Date of the request : 26-Nov-1990
Batch : RHE15 Page : 3/3

QUALITY CONTROL

Decision : RELEASED
Remarks
not for human use

Analyst in charge of study : Pascal CARDON 07-Dec-1990

Head of Quality Control : Gerard MAIRE

COMPLEMENTARY RESULTS

SR 29010
Analysis n° : 2775 Date of the request : 26-Nov-1990
Batch : RHE15 Complementary Page : 1/1

TESTS	SPECIFICATIONS	RESULTS
COMPLEMENTARIES RESULTS		
TESTS		
Residual oxygen content	not more than 2.0 per cent	0.5 per cent (1)
ASSAY		
Biological activity (on the substance as it is)	13060 to 15960 AIIa U. per mg	not performed

TABLE 26

AMINO ACID COMPOSITION OF VARIOUS RECOMBINANT HIRUDIN PREPARATIONS AS DETERMINED BY PICO-TAG METHOD[a]

	HV1 Preparations[b]				HV2 Preparations[b]				
AA	Calc	Ciba	HHF	Knoll	Calc	Hoech	Sanofi	HPF	F1[c] - F2[c]
Asp	9	6.4	8.3	5.1	9	8.5	8.5	6.5	1.1 - 3.7
Glu	13	10.8	12.6	10.1	11	13.6	11.7	8.6	1.4 - 4.4
Ser	4	4.3	4.3	5.9	4	4.5	4.9	4.2	1.3 - 2.1
Gly	9	9.6	10.4	11.3	10	11	12.5	11.4	2.2 - 4.4
His	1	1.1	1.1	1.1	1	1.1	1.1	1.4	1.2 - 1.9
Arg	0	0	0	0.8	0	0	0	0	0.5 - 0.4
Thr	4	4.1	3.9	3.6	5	5.2	5.1	4.2	0.8 - 2.2
Ala	0	0	0	1.6	0	0	0	1.6	1.4 - 1.3
Pro	3	3.5	3.6	3.4	3	3.6	3.6	5.8	1.0 - 2.2
Tyr	2	2.0	2.0	2.0	2	2.0	2.0	2.0	0.6 - 1.1
Val	4[de]	(3.3)	(2.7)	(3.0)	2	2.0	2.2	1.7	1.4 - 1.7
Met	0	0	0	0	0	0	0	0	0.0 - 0.0
Cys	6[df]	(2.9)	(4.6)	(2.6)	6[df]	(4.3)	(3.6)	(3.7)	(2.8 - 2.0)
Ile	2	2.0	1.9	1.9	3	1.8	3.0	2.2	0.8 - 1.5
Leu	4	4.0	3.9	3.8	4	4.8	4.3	2.7	1.2 - 1.9
Phe	1	1.3	1.2	1.4	1	1.3	1.2	1.4	1.0 - 1.0
Lys	3	3.4	3.2	2.6	4	3.0	3.9	3.3	2.1 - 4.8

a-Analyses that do not agree with the calculated values within ±20% are underlined. b-The N-terminal sequences, i.e., VVTY of HV-1 and ITYT of rHV2, were checked by using an ABI 471 A protein/peptide sequencer. c-Fraction 1 and 2 isolated by HPLC from Biopharm's preparation as its two main components being present in a ratio of about 25:75. d-Under the conditions of acid hydrolysis used, the val-val of HV1 does not split completely and a partial decomposition of cys takes place, thus the values obtained for [e]val and [f]cys are uncertain.

This is an excerpt from a letter from Dr. Sandor Bajusz, Institute for Drug Research, dated October 29, 1991, addressed to Dr. Jawed Fareed, Director, Hemostasis Research Laboratories, Loyola University Medical Center. Reprinted, by permission.

APPENDIX III

COPYRIGHT PERMISSION FORMS

Permission was obtained from authors of publications from which excerpts were reproduced or modified. The letters of permission obtained from each author are contained in the following pages.

LOYOLA
UNIVERSITY
CHICAGO

LOYOLA UNIVERSITY MEDICAL CENTER

2160 South First Avenue
Maywood, Illinois 60153
Telephone: (708) 216-9000

January 30, 1995

Lalitha Iyer

Tel: (708) 216-3262
Fax: 708-216-6660

Ms. Alina Ozimek,
The Yale Medical Historical Library,
333, Cedar Street,
P.O. Box 3333,
New Haven, CT 06510.

Dear Ms. Ozimek,

I am completing a doctoral dissertation at the department of Pharmacology, Loyola University Chicago entitled "Pharmacokinetics and Pharmacodynamics of Recombinant Hirudin Variant 2 (rHV2) in Animal Models". I would like your permission to modify and reproduce in my dissertation an excerpt from the following:

An illustration of the 1827 lithograph showing a physician placing leeches on patient's neck. Enclosed please find a copy of this illustration, as reproduced in my dissertation.

The requested permission extends to any future revisions and editions of my dissertation, including non-exclusive world rights in all languages, and to the prospective publication of my dissertation by University Microfilms, Inc. These rights will in no way restrict republication of the material in any other form by you or by others authorized by you. Your signing of this letter will also confirm that you own the copyright to the above-described material.

If these arrangements meet with your approval, please sign this letter where indicated below and return it to me in the enclosed return envelope. I would really appreciate it if could please send the approval by February 10, 1995.

Thank you very much.

Sincerely,

Lalitha Iyer,
Dept. of Pharmacology,
Loyola University Chicago.

PERMISSION GRANTED FOR THE
USE REQUESTED ABOVE:

( )

Date: 2/1/95

ciba

Pharmaceutical Division

GESENDET

Ciba-Geigy Limited
CH-4002 Basle
Switzerland

Dr. W. Märki
Core Drug Discovery Techn.
PH 2.251
K-681.3.43
Tel. (+61)696.55.84
Fax (+61)696.40.69

Lalitha Iyer
Dept. of Pharmacology
Loyola University Chicago
Medical Center
2160 South First Avenue
Maywood, Illinois 216-9000 / U.S.A.

FAX: 708-216-6660

Basel, January 27, 1995

Your letter to Dr. R. Wallis, January 20, 1995
e.g. Copyright request on enclosed illustration

Dear Mrs. Iyer,

On request of Dr. Wallis from Biopharm (U.K.) Ltd. I give you the permission to include / use the enclosed figure on the primary structures of hirudin isoinhibitors in your doctoral dissertation.

I hope to have been of assistance to you.

With kind regards,

Märki

W. Märki

Telefax

Schattauer
F. K. Schattauer Verlagsgesellschaft mbH
Medizin und Naturwissenschaften
Stuttgart - New York

Empfänger Receiver	Anzahl der Seiten einschl. dieser: number of pages including this one:
Lalitha Iyer Dept.of Pharmacology Loyola University Chicago Maywood, IL Fax: 001-708-216-6660	Telefax (07 11) 2 29 87 50 Telefon (07 11) 2 29 87- Phone
	Datum Date February 17,1995/he

Dear Mrs.Iyer:

Thank you for your letter of February 11,1995, received by fax. We grant you permission to reproduce the modified illustration (fig.1) of the article by Märki and Wallis "The Anticoagulant and Antithrombotic Properties of Hirudins", published in Thrombosis and Haemostasis 64 (3): 344-348 in your doctoral dissertation.

Please give appropriate credit to original publication in a standard credit line.

Yours sincerely,

H. Hansen

Hannelore Hansen
Manager Thrombosis and Haemostasis

LOYOLA
UNIVERSITY
CHICAGO

2160 South First Avenue
Maywood, Illinois 60153
Telephone: (708) 216-3261
Fax: (708) 216-6596

LOYOLA UNIVERSITY MEDICAL CENTER
STRITCH SCHOOL OF MEDICINE
Department of Pharmacology & Experimental Therapeutics

January 11, 1995

Lalitha Iyer

Tel: (708) 216-3262
Fax: 708-216-6660

A. Tulinsky, Ph.D.,
Dept. of Chemistry,
Michigan State University,
320, Chemistry Bldg.,
East Lansing, MI 48824-1322.

Dear Dr. Tulinsky,

This letter will confirm my conversation with your secretary. I am completing a doctoral dissertation at the department of Pharmacology, Loyola University Chicago entitled "Pharmacokinetics and Pharmacodynamics of Recombinant Hirudin Variant 2 (rHV2) in Animal Models". I would like your permission to modify and reproduce in my dissertation, an excerpt from the following:

> Rydel, T.J., Ravichandran, K.G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C., and Fenton II, J.W. 1990. The structure of a complex of recombinant hirudin and human α-thrombin. *Science*. 249:277-280.

The excerpt to be reproduced is an illustration on the sequence of recombinant hirudin variant 2-Lys^{47} on page 278 (figure 1) of the above mentioned article. I have reproduced the sequence of native hirudin alogside this figure, indicating differences between native and recombinant hirudin in the "Review of Literature" chapter of my dissertation. Enclosed please find a copy of this illustration as modified in my dissertation.

The requested permission extends to any future revisions and editions of my dissertation, including non-exclusive world rights in all languages, and to the prospective publication of my dissertation by University Microfilms, Inc. These rights will in no way restrict republication of the material in any other form by you or by others authorized by you. Your signing of this letter will also confirm that you own the copyright to the above-described material.

If these arrangements meet with your approval, please sign this letter where indicated below and return it to me in the enclosed return envelope.

Thank you very much.

Sincerely,

Lalitha Iyer,
Dept. of Pharmacology,
Loyola University Chicago.

PERMISSION GRANTED FOR THE
USE REQUESTED ABOVE:

A. Tulin

( )

Date: Jan. 17, 1995.

LOYOLA
UNIVERSITY
CHICAGO

2160 South First Avenue
Maywood, Illinois 60153
Telephone: (708) 216-3261
Fax: (708) 216-6596

LOYOLA UNIVERSITY MEDICAL CENTER
STRITCH SCHOOL OF MEDICINE
Department of Pharmacology & Experimental Therapeutics

January 20, 1995

Lalitha Iyer

Tel: (708) 216-3262
Fax: 708-216-6660

Claudine Picard, Ph.D.,
Service Analyse de Reserche,
Analytic Research,
Sanofi Reserche,
195 route d'Espagne,
B.P. 1169, 31036 Toulouse Cedex,
France.

Dear Dr. Picard,

This letter will confirm our recent fax messages. As you know, I am completing a doctoral dissertation at the department of Pharmacology, Loyola University Chicago entitled "Pharmacokinetics and Pharmacodynamics of Recombinant Hirudin Variant 2 (rHV2) in Animal Models". I would like your permission to modify and reproduce in my dissertation an excerpt from the following:

Tuong et al., 1992. Characterization of the deamidated forms of recombinant hirudin. *Biochemistry*. 31:8291-8299.

The excerpt to be reproduced is an illustration on the primary structure of recombinant hirudin variant 2 (rHV2-Lys 47) that appears on page 8291 (figure 1) of the above article. Enclosed please find a copy of this illustration, as modified in my dissertation.

The requested permission extends to any future revisions and editions of my dissertation, including non-exclusive world rights in all languages, and to the prospective publication of my dissertation by University Microfilms, Inc. These rights will in no way restrict republication of the material in any other form by you or by others authorized by you. Your signing of this letter will also confirm that you own the copyright to the above-described material.

If these arrangements meet with your approval, please sign this letter where indicated below and return it to me in the enclosed return envelope. I would really appreciate it if could please send the approval by January 31, 1995.

Thank you very much.

Sincerely,

Lalitha Iyer,
Dept. of Pharmacology,
Loyola University Chicago.

PERMISSION GRANTED FOR THE
USE REQUESTED ABOVE:

Picard

(C. PICARD)

Date: 26/01/95

LOYOLA
UNIVERSITY
CHICAGO

2160 South First Avenue
Maywood, Illinois 60153
Telephone: (708) 216-3261
Fax: (708) 216-6596

LOYOLA UNIVERSITY MEDICAL CENTER
STRITCH SCHOOL OF MEDICINE
Department of Pharmacology & Experimental Therapeutics

January 11, 1995

Lalitha Iyer

Tel: (708) 216-3262
Fax: 708-216-6660

Professor Fritz Markwardt,
Institute of Pharmacology and Toxicology
Medical Academy Erfurt,
Erfurt,
Germany.

Dear Prof. Markwardt,

This letter will confirm my recent telephone conversation with Prof. Breddin. I am completing a doctoral dissertation at the department of Pharmacology, Loyola University Chicago entitled "Pharmacokinetics and Pharmacodynamics of Recombinant Hirudin Variant 2 (rHV2) in Animal Models". I would like your permission to modify and reproduce in my dissertation an excerpt from the following:

Markwardt, 1991. Past, present and future of hirudin. *Haemostasis*. 21(suppl. 1):11-26.

The excerpt to be reproduced is an illustration on the scheme of hirudin-thrombin reaction that appears on page 13 (figure 1) of the above article. Enclosed please find a copy of this illustration, as modified in my dissertation.

The requested permission extends to any future revisions and editions of my dissertation, including non-exclusive world rights in all languages, and to the prospective publication of my dissertation by University Microfilms, Inc. These rights will in no way restrict republication of the material in any other form by you or by others authorized by you. Your signing of this letter will also confirm that you own the copyright to the above-described material.

If these arrangements meet with your approval, please sign this letter where indicated below and return it to me in the enclosed return envelope. I would really appreciate it if could please send the approval by February 10, 1995.

Thank you very much.

Sincerely,

Lalitha Iyer,
Dept. of Pharmacology,
Loyola University Chicago.

PERMISSION GRANTED FOR THE
USE REQUESTED ABOVE:

( )

Date: 2.2.95

LOYOLA
UNIVERSITY
CHICAGO

2160 South First Avenue
Maywood, Illinois 60153
Telephone: (708) 216-3261
Fax: (708) 216-6596

LOYOLA UNIVERSITY MEDICAL CENTER
STRITCH SCHOOL OF MEDICINE
Department of Pharmacology & Experimental Therapeutics

February 20, 1995

Lalitha Iyer

Tel: (708) 216-3262
Fax: 708-216-6660

Jawed Fareed, Ph.D.,
Professor of Pathology and Pharmacology,
Loyola University Medical Center,
2160, S.First Avenue,
Maywood, IL 60153.

Dear Dr. Fareed,

This letter will confirm our recent conversation. As you know, I am completing a doctoral dissertation at the department of Pharmacology, Loyola University Chicago entitled "Pharmacokinetics and Pharmacodynamics of Recombinant Hirudin Variant 2 (rHV2) in Animal Models". I would like your permission to modify and reproduce in my dissertation, an excerpt from the following:

> Fareed *et al.*, 1991b, "An objective perspective on recombinant hirudin: a new anticoagulant and antithrombotic agent", *Blood Coag. Fibrinol.*, 2:135-147.

The excerpt to be reproduced is a table on the laboaratory assays for measurement of hirudin, that appears on page 138 of the above mentioned article. I have reproduced the sequence of native hirudin alogside this figure, indicating differences between native and recombinant hirudin in the "Review of Literature" chapter of my dissertation. Enclosed please find a copy of this illustration as modified in my dissertation.

The requested permission extends to any future revisions and editions of my dissertation, including non-exclusive world rights in all languages, and to the prospective publication of my dissertation by University Microfilms, Inc. These rights will in no way restrict republication of the material in any other form by you or by others authorized by you. Your signing of this letter will also confirm that you own the copyright to the above-described material.

If these arrangements meet with your approval, please sign this letter where indicated below and return it to me in the enclosed return envelope.

Thank you very much.

Sincerely,

Lalitha Iyer,
Dept. of Pharmacology,
Loyola University Chicago.

PERMISSION GRANTED FOR THE
USE REQUESTED ABOVE:

( )

Date: 2/22/95

LOYOLA
UNIVERSITY
CHICAGO

2160 South First Avenue
Maywood, Illinois 60153
Telephone: (708) 216-3261
Fax: (708) 216-6596

LOYOLA UNIVERSITY MEDICAL CENTER
STRITCH SCHOOL OF MEDICINE
Department of Pharmacology & Experimental Therapeutics

January 11, 1995

Lalitha Iyer

Tel: (708) 216-3262
Fax: 708-216-6660

Jeanine Walenga, Ph.D.,
Associate Professor,
Department of Pathology &
Thoracic and Cardiovascular Surgery,
Loyola University Medical Center,
2160, S. First Avenue,
Maywood, IL 60153.

Dear Dr. Walenga,

This letter will confirm our recent conversation. I am completing a doctoral dissertation at the department of Pharmacology, Loyola University Chicago entitled "Pharmacokinetics and Pharmacodynamics of Recombinant Hirudin Variant 2 (rHV2) in Animal Models". I would like your permission to modify and reproduce in my dissertation an excerpt from the following:

Walenga, 1987. Factor X_a inhibition in mediating antithrombotic actions: application of a synthetic heparin pentasaccharide. Ph.D. diss., University of Paris.

The excerpt to be reproduced is an illustration on the modified rabbit jugular vein stasis thrombosis model on page number 82 of your Ph.D. dissertation. Enclosed please find a copy of this illustration as modified in my dissertation.

The requested permission extends to any future revisions and editions of my dissertation, including non-exclusive world rights in all languages, and to the prospective publication of my dissertation by University Microfilms, Inc. These rights will in no way restrict republication of the material in any other form by you or by others authorized by you. Your signing of this letter will also confirm that you own the copyright to the above-described material.

If these arrangements meet with your approval, please sign this letter where indicated below and return it to me in the enclosed return envelope.

Thank you very much.

Sincerely,

Lalitha Iyer,
Dept. of Pharmacology,
Loyola University Chicago.

PERMISSION GRANTED FOR THE
USE REQUESTED ABOVE:

Jasmine Wakenya

(Jasmine Wakenya)

Date: January 12, 1995

LOYOLA
UNIVERSITY
CHICAGO

2160 South First Avenue
Maywood, Illinois 60153
Telephone: (708) 216-3261
Fax: (708) 216-6596

LOYOLA UNIVERSITY MEDICAL CENTER
STRITCH SCHOOL OF MEDICINE
Department of Pharmacology & Experimental Therapeutics

January 11, 1995

Lalitha Iyer

Tel: (708) 216-3262
Fax: 708-216-6660

Jeanine Walenga, Ph.D.,
Associate Professor,
Department of Pathology &
Thoracic and Cardiovascular Surgery,
Loyola University Medical Center,
2160, S. First Avenue,
Maywood, IL 60153.

Dear Dr. Walenga,

This letter will confirm our recent conversation. I am completing a doctoral dissertation at the department of Pharmacology, Loyola University Chicago entitled "Pharmacokinetics and Pharmacodynamics of Recombinant Hirudin Variant 2 (rHV2) in Animal Models". I would like your permission to modify and reproduce in my dissertation, an excerpt from the following:

Walenga, 1987. Factor X_a inhibition in mediating antithrombotic actions: application of a synthetic heparin pentasaccharide. Ph.D. diss., University of Paris.

The excerpt to be reproduced is an illustration on the clot-score grading system in the modified rabbit jugular vein stasis thrombosis model on page number 83 of your Ph.D. dissertation. Enclosed please find a copy of this illustration as modified in my dissertation.

The requested permission extends to any future revisions and editions of my dissertation, including non-exclusive world rights in all languages, and to the prospective publication of my dissertation by University Microfilms, Inc. These rights will in no way restrict republication of the material in any other form by you or by others authorized by you. Your signing of this letter will also confirm that you own the copyright to the above-described material.

If these arrangements meet with your approval, please sign this letter where indicated below and return it to me in the enclosed return envelope.

Thank you very much.

Sincerely,

Lalitha Iyer,
Dept. of Pharmacology,
Loyola University Chicago.

PERMISSION GRANTED FOR THE
USE REQUESTED ABOVE:

Jeanine Walenga

(Jeanine Walenga)

Date: January 12, 1995

LOYOLA
UNIVERSITY
CHICAGO

LOYOLA UNIVERSITY MEDICAL CENTER

2160 South First Avenue
Maywood, Illinois 60153
Telephone: (708) 216-9000

January 16, 1995

Lalitha Iyer

Tel: (708) 216-3262
Fax: 708-216-6660

Adrienne Racanelli, Ph.D,
DuPont Merck Pharmaceutical Company,
Experimental Station Bldg. E400/3213,
Wilmington, DE 19880-0400.

Dear Dr. Racanelli,

This letter will confirm our recent telephone messages. I am completing a doctoral dissertation at the department of Pharmacology, Loyola University Chicago entitled "Pharmacokinetics and Pharmacodynamics of Recombinant Hirudin Variant 2 (rHV2) in Animal Models". I would like your permission to modify and reproduce in my dissertation an excerpt from the following:

Racanelli, 1990. Biochemical and pharmacological studies on the interaction of protamine with heparins. Ph.D. diss., Loyola University Chicago.

The excerpt to be reproduced is an illustration on the modified rabbit ear blood loss model on page number 332 of your Ph.D. dissertation. Enclosed please find a copy of this illustration, as modified in my dissertation.

The requested permission extends to any future revisions and editions of my dissertation, including non-exclusive world rights in all languages, and to the prospective publication of my dissertation by University Microfilms, Inc. These rights will in no way restrict republication of the material in any other form by you or by others authorized by you. Your signing of this letter will also confirm that you own the copyright to the above-described material.

If these arrangements meet with your approval, please sign this letter where indicated below and return it to me in the enclosed return envelope.

Thank you very much.

Sincerely,

Lalitha Iyer,
Dept. of Pharmacology,
Loyola University Chicago.

PERMISSION GRANTED FOR THE
USE REQUESTED ABOVE:

Adrienne Racanelli, PhD
(Adrienne Racanelli, PhD)

Date: January 24, 1994

LOYOLA
UNIVERSITY
CHICAGO

2160 South First Avenue
Maywood, Illinois 60153
Telephone: (708) 216-3261
Fax: (708) 216-6596

LOYOLA UNIVERSITY MEDICAL CENTER
STRITCH SCHOOL OF MEDICINE
Department of Pharmacology & Experimental Therapeutics

March 27, 1995

Lalitha Iyer

Tel: (708) 216-3262
Fax: 708-216-6660

Sandor Bajusz, Ph.D.,
Institute for Drug Research,
H-1325, Budapest, POB 82,
Szabadsagharcosok u. 47-49,
Hungary.

Dear Dr. Bajusz,

This letter will confirm our recent conversation. I am completing a doctoral dissertation at the department of Pharmacology, Loyola University Chicago entitled "Pharmacokinetics and Pharmacodynamics of Recombinant Hirudin Variant 2 (rHV2) in Animal Models". I would like your permission to modify and reproduce in my dissertation an excerpt from the following:

A letter written by you, addressed to Dr. Jawed Fareed, Director, Hemostasis Research Laboratories, Loyola University Medical Center, dated October 29, 1991.

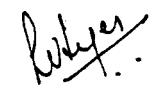
The excerpt to be reproduced is a table listing the amino acid composition of different variants of hirudin. Enclosed please find a copy of this table as modified in my dissertation.

The requested permission extends to any future revisions and editions of my dissertation, including non-exclusive world rights in all languages, and to the prospective publication of my dissertation by University Microfilms, Inc. These rights will in no way restrict republication of the material in any other form by you or by others authorized by you. Your signing of this letter will also confirm that you own the copyright to the above-described material.

If these arrangements meet with your approval, please sign this letter where indicated below and return it to me in the enclosed return envelope.

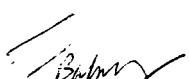
Thank you very much.

Sincerely,



Lalitha Iyer,
Dept. of Pharmacology,
Loyola University Chicago.

PERMISSION GRANTED FOR THE
USE REQUESTED ABOVE:



(S. BAJUSZ)

Date: March 27, 1995

LOYOLA
UNIVERSITY
CHICAGO

2160 South First Avenue
Maywood, Illinois 60153
Telephone: (708) 216-3261
Fax: (708) 216-6596

LOYOLA UNIVERSITY MEDICAL CENTER
STRITCH SCHOOL OF MEDICINE
Department of Pharmacology & Experimental Therapeutics

March 27, 1995

Lalitha Iyer

Tel: (708) 216-3262
Fax: 708-216-6660

Jawed Fareed, Ph.D.,
Professor of Pathology and Pharmacology,
Director, Hemostasis Research Laboratories,
Loyola University Medical Center,
2160, S. First Avenue,
Maywood, IL 60153.

Dear Dr. Fareed,

This letter will confirm our recent conversation. I am completing a doctoral dissertation at the department of Pharmacology, Loyola University Chicago entitled "Pharmacokinetics and Pharmacodynamics of Recombinant Hirudin Variant 2 (rHV2) in Animal Models". I would like your permission to modify and reproduce in my dissertation an excerpt from the following:

A letter written by Dr. Sandor Bajusz, Institute for Drug Research, Budapest, Hungary, dated October 29, 1991, and addressed to you.

The excerpt to be reproduced is a table listing the amino acid composition of different variants of hirudin. Enclosed please find a copy of this table as modified in my dissertation.

The requested permission extends to any future revisions and editions of my dissertation, including non-exclusive world rights in all languages, and to the prospective publication of my dissertation by University Microfilms, Inc. These rights will in no way restrict republication of the material in any other form by you or by others authorized by you. Your signing of this letter will also confirm that you own the copyright to the above-described material.

If these arrangements meet with your approval, please sign this letter where indicated below and return it to me in the enclosed return envelope.

Thank you very much.

Sincerely,

Lalitha Iyer,
Dept. of Pharmacology,
Loyola University Chicago.

PERMISSION GRANTED FOR THE
USE REQUESTED ABOVE:

(JAWED FAREED)

Date: April 1, 1995

REFERENCES

Abel, J.J., Rowntree, L.G. and Turner, B.B. 1914. On the removal of diffusible substances from the circulating blood of living animals by dialysis. *Jour. Pharmacol. Exptl. Therap.* **5**:275-318.

Adams, S.L. 1989. The medicinal leech: historical perspectives. *Sem. Thromb. Hemost.* **15**(3):261-264.

Alving, A.S. and Miller, B.F. 1940. A practical method for the measurement of glomerular filtration rate (inulin clearance) with an evaluation of the clinical significance of this determination. *Arch. Int. Med.* **66**:306-318.

Amiral, J., Cluzeau, D., Adam, M. and Chambrette, B. 1991. Measurement of hirudin on plasma and other biological fluids by a two site immunoassay. *Thromb. Haemost.* **65**(6):1289.

Antman, E.M. for the TIMI 9A investigators. 1994. Hirudin in myocardial infarction. Safety report from the thrombolysis and thrombin inhibition in myocardial infarction (TIMI) 9A trial. *Circulation*. **90**:1624-1630.

Aylward, P. 1993. Gusto II trial. *Aust. NZ Jour. Med.* **23**:766-768.

Bacher, P., Walenga, J.M., Iqbal, O., Bajusz, S., Breddin, K. and Fareed, J. 1993. The antithrombotic and anticoagulant effects of a synthetic tripeptide and recombinant hirudin in various animal models. *Thromb. Res.* **71**:251-263.

Bagdy, D., Barabas, E. and Graf, L. 1973. Large scale preparation of hirudin. *Thromb. Res.* **2**:229-238.

Barnard, H.F., Bassir, O. and Hough, J.M. 1955. Fall in the inulin clearance following a single injection. *Q. Jour. Exptl. Physiol.* **40**:217-224.

Baskova, I.P., Khalil, S., Nikonov, G.J., Rabinovich, S.E., Nartikova, V.F. and Paskhina, T.S. 1981. Inhibition of human blood plasma kallikrein by salivary gland secretion and an extract of the medicinal leech. *Biokhimiya*. **53**(9):1467-1473.

Baskova, I.P., Cherkesova, D.V. and Mosolov, V.V. 1983. Hirudin from leech heads and whole leeches and "pseudo-hirudin" from bodies. *Thromb. Res*. **30**:459-467.

Baskova, I.P., Yusupova, G.I. and Nikonov, G.I. 1984. Lipase and cholesterol esterase activity of salivary gland secretion of the leech *Hirudo medicinalis*. *Biokhimiya*. **49**(4):676-678.

Bauer, K.A. 1994. Hypercoagulability - a new cofactor in the protein C anticoagulant pathway. *The New Engld. Jour. Med.* **330**(8):566-567.

Bergmann, C., Dodt, J., Kohler, S., Fink, E. and Gassen, H.G. 1986. Chemical synthesis and expression of a gene coding for hirudin, the thrombin-specific inhibitor from the leech *Hirudo medicinalis*. *Biol. Chem. Hoppe Seyler*. **367**:731-740.

Berscheid, G., Grötsch, H., Neubauer, H., Pünter, J., Reindl, J. and Seipp, P. 1992. Determination of rDNA and A-human thrombin-hirudin complex in plasma samples: enzyme linked immunosorbent assays for hirudin and complex vs. chromogenic thrombin substrate assay. *Thromb. Res*. **66**:33-42.

Bichler, J., Fichtl, B., Siebeck, M. and Fritz, H. 1988. Pharmacokinetics and pharmacodynamics of hirudin in man after single subcutaneous and intravenous bolus administration. *Arzneim.-Forsch./Drug Res*. **38**(5):704-710.

Bichler, J., Gimmerli, R. and Bichler, H. 1991. Studies for revealing a possible sensitization to hirudin after repeated intravenous injections in baboons. *Thromb. Res.* **61**:39-51.

Bichler, J., Baynes, J.W. and Thorpe, S. 1993. Catabolism of hirudin and thrombin-hirudin complexes in the rat. *Biochem. Jour.* **296**:771-776.

Bick, R.L. 1992a. Hereditary coagulation protein defects. In *Disorders of Thrombosis and Hemostasis*, ed. K.D. Johnson, 109-136, Chicago: American Society of Clinical Pathologists Press.

Bick, R.L. 1992b. Hypercoagulability and Thrombosis. In *Disorders of Thrombosis and Hemostasis*, ed. K.D. Johnson, 261-289, Chicago: American Society of Clinical Pathologists Press.

Bjornsson, T.D. and Levy, G. 1979a. Pharmacokinetics of heparin. I. studies of dose dependence in rats. *Jour. Pharmacol. Exptl. Therap.* **210**(2):237-242.

Bjornsson, T.D. and Levy, G. 1979b. Pharmacokinetics of heparin. II. studies of time dependence in rats. *Jour. Pharmacol. Exptl. Therap.* **210**(2):243-246.

Braun, P.J. 1990. Binding properties of hirudin determined by gel filtration and gel electrophoresis. *Thromb. Res.* **59**:657-662.

Broze, G.J. Jr. 1992. The role of tissue factor pathway inhibitor in a revised coagulation cascade. *Sem. Hematol.* **29**(3):159-169.

Broze, G.J. Jr. 1994b. The tissue factor pathway of coagulation:factor VII, tissue factor, and tissue factor pathway inhibitor. In: *Haemostasis and Thrombosis*, ed. A. L. Bloom, C. D. Forbes, D. P. Thomas and G. D. Tuddenham, 349-377, 3d ed., vol. I. Edinburgh, London, Madrid, Melbourne, New York, Tokyo: Churchill Livingstone.

Broze, G.J. Jr., Warren, L.A., Novotny, W.F., Higuchi, D.A., Girard, J.J. and Miletich, J.P. 1988. The lipoprotein-associated coagulation inhibitor that inhibits the factor VII-tissue factor complex also inhibits factor X_a: Insight into its possible mechanism of action. *Blood*. **71**:335-343.

Bruggener, E., Walsmann, P. and Markwardt, F. 1989. Neutralization of hirudin anticoagulant action by DIP-thrombin. *Pharmazie*. **44**:648-649.

Bucha, E., Markwardt, F. and Nowak, G. 1990. Hirudin in hemodialysis. *Thromb. Res.* **60**:445-455.

Butler, K.D., Dolan, S.L., Talbot, M.D. and Wallis, R.B. 1993. Factor VIII and DDAVP reverse the effect of recombinant desulfatohirudin (CGP-39393) on bleeding in the rat. *Blood Coag. Fibrinol.* **4**:459-464.

Byron, P.R. and Notari, R.E. 1976. Critical analysis of "flip-flop" phenomenon in two-compartment pharmacokinetic model. *Jour. Pharm. Sci.* **65**(8):1140-1144.

Cade, J.F., Buchanan, M.R., Boneu, B., Ockelford, P., Carter, C. J., Cerkus, A.L. and Hirsh, J. 1984. A comparison of the antithrombotic and hemorrhagic effects of low molecular weight heparin fractions: the influence of the method of preparation. *Thromb. Res.* **35**:613-625.

Camici, M. and Evangelisti, L. 1993. Monitoring heparin therapy. *Minerva Medica*. **84**(10):507-509.

Chan, K.K. and Gibaldi, M. 1985. Assessment of drug absorption after oral administration. *Jour. Pharm. Sci.* **74**(4):388-393.

Chang, J.: 1983. The functional domain of hirudin, a thrombin-specific inhibitor. *FEBS Letters*. **164**(2):307-313.

Chang, J.: 1991. Stability of hirudin, a thrombin-specific inhibitor: the structure of alkaline-inactivated Hirudin. *Jour. Biol. Chem.* **266**(17):10839-10843.

Chang, J-Y., Grossenbacher, H., Meyhack, B. and Märki, W. 1993. Production of disulfide-linked hirudin dimer by in vitro folding. *FEBS Letters*. **336**(1):53-56, 1993.

Chaykin, S. 1966. Methods for protein determination (with introductory remarks concerning photometry in general). In *Biochemistry Laboratory Techniques*. New York, London, Sydney: J. Wiley & sons, Inc.

Chesebro, J.H., Badimon, L. and Fuster, V. 1991a. Importance of antithrombin therapy during coronary angioplasty. *Jour. Amer. Coll. Cardiol.* **17**(6):96B-100B.

Chesebro, J.H., Zoldhelyi, P., Badimon, L. and Fuster, V. 1991b. Role of thrombin in arterial thrombosis: implications for therapy. *Thromb. Haemost.* **66**(1):1-5.

Cochrane, C.G., Revak, S.D. and Weupper, K.D. 1973. Activation of Hageman factor in solid and fluid phases. A critical role of kallikrein. *Jour. Exp. Med.* **138**:1564-1583.

Coniff, R. 1987. The little suckers have made a comeback. *Discover*. 85-94.

Courtney, M., Loison, G., Lemoine, Y., Riehl-Bellon, N., Degryse, E., Brown, S.W., Cazenave, J.-P., Defreyn, G., Delebassee, D., Bernat, A., Maffrand, J.-P. and Roitsch, C. 1989. Production and evaluation of recombinant hirudin. *Sem. Thromb. Hemost.* **15**(3):288-292.

Dahlback, B. and Hildebrand, B. 1994. Inherited resistance to activated protein C is corrected by anticoagulant cofactor activity found to be a property of factor V. *Proc. Natl. Acad. Sci. USA.* **91**(4):1396-1400.

Dahlback, B. and Stenflo, J. 1994. A natural anticoagulant pathway:protein C, S, C4b-binding protein and thrombomodulin. In: *Haemostasis and Thrombosis*, ed. A. L. Bloom, C. D. Forbes, D. P. Thomas and G. D. Tuddenham, 671-698, 3d ed., vol. I. Edinburgh, London, Madrid, Melbourne, New York, Tokyo: Churchill Livingstone.

Davie, E.W. and Ratnoff, O.D. 1964. Waterfall sequence for intrinsic blood clotting. *Science.* **145**:1310-1312.

Davie, E.W., Fujikawa, K., Kurachi, K. and Kisiel, W. 1979. The role of serine proteases in the blood coagulation cascade. *Adv. Enzymol.* **48**:277-318.

Davie, E.W., Fujikawa, K. and Kisiel, W. 1991. The coagulation cascade: initiation, maintenance, and regulation. *Biochemistry.* **30**(43):10363-10370.

Didisheim, P., Hattori, K. and Lewis, J.H. 1959. Hematologic and coagulation studies in various animal species. *Jour. Lab. Clin. Med.* **53**(6):866-875.

Diehl, K.-H., Romisch, B., Hein, B., Jessel, A. and Ronneberger, H. 1994. Potential hirudin antidote: investigation of an activated prothrombin complex concentrate in animal models. In *Annals of Hematology: Proceedings of the 3rd Annual Meeting in Munich, February 16-19, 1994*, by the German Society of Thrombosis and Hemostasis. **68**(II):A86.

Dodt, J. 1984: Hirudine, die Thrombininhibitoren des Blutegels "*Hirudo medicinalis*". Ph.D. diss., University of Munich, 1984.

Dodt, J., Muller, H.-P., Seemuller, U. and Chang, J. 1984: The complete amino acid structure of hirudin, a thrombin specific inhibitor. *FEBS Letters.* **165**(2):180-183.

Dodt, J., Seemuller, U., Maschler, R. and Fritz, H. 1985: The complete covalent structure of hirudin: localization of the disulfide bonds. *Biol. Chem. Hoppe Seyler.* **366**:379-385.

Dodt, J., Machleidt, W., Seemuller, V., Maschler, R. and Fritz, H. 1986. Isolation and characterization of hirudin isoinhibitors and sequential analysis of hirudin PA. *Biol. Chem. Hoppe-Seyler.* **367**:803-811.

Dodt, J., Kohler, S. and Baici, A. 1988. Interaction of site specific hirudin variants with α-thrombin. *FEBS Letters.* **229**(1):87-90.

Doutremepuich, C., Deharo, E., Guyot, M., Lalanne, M.C., Walenga, J. and Fareed, J. 1989. Antithrombotic activity of recombinant hirudin in the rat: A comparative study with heparin. *Thromb. Res.* **54**:435-445.

Doutremepuich, C., Lalanne, M.C., Doutremepuich, F., Walenga, J., Fareed, J. and Breddin, H.K. 1991. Comparative study of three recombinant hirudins with heparin in an experimental venous thrombosis model. *Hemostasis.* **21**(suppl. 1):99-106.

Emanuele, R.M. 1987. The effect of molecular weight of the anticoagulant, antiprotease, antithrombotic and pharmacokinetic actions of heparin. Ph.D. diss., Loyola University Chicago.

Fareed, J. 1994. Current perspectives on the development of synthetic and recombinant antithrombin agents. In *Proceedings of the Fifth Annual Symposium on Advances in Anticoagulant, Antithrombotic and Thrombolytic Therapeutics, in Boston, October 24-25, 1994*, by International Business Communications.

Fareed, J., Parvez, Z., Messmore, H.L., Mariani, G. and Walz, D.A. 1984. Protein C levels in activated and non-activated prothrombin complex concentrates. *Thromb. Res.* **33**:453-455.

Fareed, J., Walenga, J.M., Kumar, A. and Rock, A. 1985. A modified stasis thrombosis model to study the antithrombotic actions of heparin and its fractions. *Sem. Thromb. Hemost.* **11**(2):155-175.

Fareed, J., Walenga, J.M., Hoppensteadt, D.A. and Pifarre, R. 1989. Developmental perspectives for recombinant hirudin as an antithrombotic agent. *Biol. Clin. Hematol.* **11**:143-152.

Fareed, J., Walenga, J.M., Hoppensteadt, D., Iyer, L. and Pifarre, R. 1991a. Neutralization of recombinant hirudin: some practical considerations. *Sem. Thromb. Hemost.* **17**(2):137-144.

Fareed, J., Walenga, J.M., Iyer, L., Hoppensteadt, D. and Pifarre, R. 1991b. An objective perspective on recombinant hirudin: a new anticoagulant and antithrombotic agent. *Blood Coag. Fibrinol.* **2**:135-147.

Fenton II, J.W. 1989. Thrombin interactions with hirudin. *Sem. Thromb. Hemost.* **15**(3):265-268.

Fenton II, J.W., Villanueva, G.B., Ofosu, F.A. and Maraganore, J.M. 1991. Thrombin inhibition by hirudin: how hirudin inhibits thrombin. *Haemostasis.* **21** (Suppl. 1):27-31.

Ferguson, M.H., Olbrich, O., Robson, J.S. and Stewart, C.P. 1950. The use of inulin clearance as a measure of glomerular filtration. *Q. Jour. Exptl. Physiol.* **35**:251-279.

Fields, W.S. 1991. The history of leeching and hirudin. *Haemostasis.* **21**(Suppl. 1):3-10.

Fink, E. 1988. Proteinase inhibitors of the leech *Hirudo medicinalis*. In *Hematology: Hemostasis and Animal Venoms*. ed. H. Pirkle and F. S. Markland Jr. 307-322, vol. **7**. New York, Basel: Marcel Dekker, Inc.

Fleishaker, J.C. and Smith, R.B. 1987. Compartmental model analysis in pharmacokinetics. *Jour. Clin. Pharmacol.* **27**:922-926.

Fortkamp, E., Rieger, M., Heisterberg-Moutses, G., Schweitzer, S. and Sommer, R. 1986. Cloning and expression in *Escherichia coli* of a synthetic DNA for hirudin, the blood coagulation inhibitor in the leech. *DNA*. **5**(6):511-517.

Furutani, Y., Honjo, M., Nakajama, A., Kawamura, K., Mita, I. and Audo, K. 1988. Method for preparing a hirudin. *European Patent Application*. 0352387 A1 (priority 26.7.1988 JP 18455788).

Fuster, V. and Verstraete, M. 1991. *Thrombosis in Cardiovascular Disorders*. Philadelphia: W.B. Saunders company.

Gailani, D. and Broze, G.J. Jr. 1991. Factor XI activation in a revised model of blood coagulation. *Science*. **253**:909-912.

Gallistl, S. and Muntean, W. 1994. Thrombin-hirudin complex formation, thrombin-antithrombin-III complex formation, and thrombin generation after intrinsic activation of plasma. *Thromb. Haemost.* **72**(3):387-392.

Garcia, K.J. 1992. Lowly leech making a comeback. *The San Francisco Chronicle*. 9 September, A1 and A8.

Gibaldi, M. and Perrier, D. 1982. *Pharmacokinetics*. 2d ed. New York, Basel: Marcel Dekker, Inc.

Gibaldi, M. 1984. *Biopharmaceutics and Clinical Pharmacokinetics*. 3d ed. Philadelphia: Lea and Febiger.

Ginsberg, J.S., Nurmohamed, M.T., Gent, M., Mackinnon, B., Stevens, P., Weitz, J., Maraganore, J. and Hirsh, J. 1994. Effects on thrombin generation of single injections of Hirulog™ in patients with calf vein thrombosis. *Thromb. Haemost.* **72**(4):523-525.

Graf, L., Patthy, A., Barabas, B. and Bagdy, D. 1973. On the N-terminal residue of hirudin. *Biochim. Biophy. Acta*. **310**:416-417.

Griessbach, U., Strüzebecher, J. and Markwardt, F. 1985. Assay of hirudin in plasma using a chromogenic thrombin substrate. *Thromb. Res.* **37**(2):347-350.

Groetsch, H., Damm, D., Ben Youssef, R. and Haertel, D. 1991. Comparison of two different methods for the determination of rDNA-hirudin in plasma samples: HPLC vs. a chromogenic substrate. *Thromb. Res.* **64**:273-277.

Gusto IIa Investigators. 1994. Randomized trial of intravenous heparin versus recombinant hirudin for acute coronary syndromes. *Circulation*. **90**:1631-1637.

Gwilt, P.R. 1990. Pharmacokinetics. In *Modern Pharmacology*. ed. C.R. Craig and R.E. Stitzel. 68-81, 3d ed., Boston: Little, Brown and Company.

Hall, J.E., Guyton, A.C. and Farr, B.M. 1977. A single-injection method for measuring glomerular filtration rate. *Amer. Jour. Physiol.* **232**(1):F72-F76.

Haruyama, H. and Wuthrich, K. 1989. Conformation of recombinant desulfatohirudin in aqeous solution determined by nuclear magnetic resonance. *Biochemistry*. **28**(10):4301-4312.

Harvey, R.P., Degryse, E., Stefani, L., Schamber, F., Cazenave, J.-P., Courtney, M., Tolstochev, P. and Lecocq, J.-P. 1986. Cloning and expression of a cDNA coding for the anticoagulant hirudin from the bloodsucking leech, *Hirudo medicinalis*. *Proc. Natl. Acad. Sci. USA*. **83**:1084-1088.

Hauptmann, J. and Markwardt, F. 1992. Pharmacological aspects of the development of selective thrombin inhibitors as anticoagulants. *Sem. Thromb. Hemost.* **18**:200-217.

Haycraft, J.B. 1884. On the action of a secretion obtained from the medicinal leech on the coagulation of the blood. *Proc. Roy. Soc. Lond*. **36**:478-487.

Henschen, A., Markwardt, F. and Walsmann, P. 1988. Evidence for the identity of hirudin isolated after kidney passage with the starting material. *Folia Haematol*. **115**(1-2, Suppl):59-63.

Hill, A.V. 1910. The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves. *Jour. Physiol. (Lond.)*. **40**:iv-vii.

Ho, C.H. Wu and S.Y. 1991. The influence of time, temperature and packed cell on activated partial thromboplastin time and prothrombin time. *Thromb. Res*. **62**(6):625-633.

Hoet, B., Tornai, I., Arnout, J., Close, P., Vermylen, J. and Verstraete, M. 1994. Open study of intravenous recombinant hirudin (CGP 39393) on platelet function and coagulation in healthy volunteers. *Drug Invest.* **7**:127-133.

Holford, N.H.G. and Sheiner, L.B. 1981. Pharmacokinetic and pharmacodynamic modelling *in vivo. CRC Crit. Rev. Bioengg.* **5**:273-322.

Holford, N.H.G. and Sheiner, L.B. 1982. Kinetics of pharmacologic response. *Pharmacol. Therap.* **16**:143-166.

Hoppensteadt, D. 1989. Studies on the anticoagulant, antithrombotic and bleeding effects of dermatan, heparan and heparin. M.S. thesis, University of Illinois at Chicago.

Howarth, S. 1993. Activated partial thromboplastin time reagents: an evaluation. *Brit. Jour. Biomed. Sci.* **50**(2):109-113.

Hubbard, T., Olivier, T., Bacher, P., Walenga, J.M., Galal, H., Fareed, J. and Pifarre, R. 1991. Use of recombinant hirudin to increase the patency rate of microanastomosis in a rabbit model. *Blood Coag. Fibrinol.* **2**:101-103.

Ibbtson, S.H., Grant, P.J., Kerry, R., Findlay, V.S. and Prentice, C.R.M. 1991. The influence of infusions of 1-Desamino-8-D-Arginine Vasopressin (DDAVP) *in vivo* on the anticoagulant effect of recombinant hirudin (CGP 39393) *in vitro*. *Thromb. Haemost.* **65**:64-66.

Iyer, L. and Fareed, J. 1995. Determination of specific activity of recombinant hirudin by thrombin titration method. *Thromb. Res.* In press.

Iyer, L., Hoppensteadt, D.H., Walenga, J.M. and Fareed, J. 1990. Development and application of methods for monitoring the pharmacokinetics/ pharmacodynamics of recombinant hirudins and related agents. *The Pharmacologist*. **32**(3):185.

Iyer, L., Koza, M., Iqbal, O., Calabria, R. and Fareed, J. 1993a. Studies on the pharmacokinetics and pharmacodynamics of recombinant hirudin (rHV2-Lys 47) after intravenous and subcutaneous administration in dogs. *Thomb. Res.* **69**:259-269.

Iyer, L., Shavit, J., Koza, M., Calabria, R., Moran, S. and Fareed, J. 1993b. Alteration of pharmacokinetics and pharmacodynamics of recombinant hirudin (rHV2-Lys47) after repeated intravenous administration in dogs. *Thromb. Res.* **69**:59-70.

Iyer, L., Amiral, J., Adam, M. and Fareed, J. 1995. Development and validation of two enzyme-linked immunosorbent methods for the measurement of recombinant hirudin in biological fluids. *Sem. Thromb. Hemost.* In press.

Jackson,C.V. 1994. Preclinical and clinical pharmacology of efegatran: a novel antithrombin for the treatment of acute coronary syndromes. In *Proceedings of the Fifth Annual Symposium on Advances in Anticoagulant, Antithrombotic and Thrombolytic Therapeutics, in Boston, October 24-25, 1994,* by International Business Communications.

Jacoby, C: 1904. Uber Hirudin. *Dstch med Wscher.* **30**:1786.

Janes, M., Meyhack, B., Zimmerman, W. and Hinnen, A. 1990. The influence of GAP promoter variants on hirudin production, average plasmid copy number and cell growth in *Saccharomyces cerevisiae. Curr. Genet.* **18**:97-103.

Jeske, W. 1995. Pharmacological studies on synthetic analogues of heparin with selective affinity to endogenous serine protease inhibitors. Ph.D. dissertation, Loyola University Chicago. *In Preparation.*

Johnson, P.H. 1994. Hirudin: Clinical potential of a thrombin inhibitor. *Annu. Rev. Med.* **45**:165-177.

Johnson, P.H., Sze, P., Winant, R., Payne, P. and Lazar, J.B. 1989. Biochemistry and genetic engineering of hirudin. *Sem. Thromb. Hemost.* **15**(3):302-315.

Kaczmarczyk, G. and Reinhardt, H.W. 1975. Arterial blood gas tensions and acid-base status of Wistar rats during thiopental and halothane anesthesia. *Lab. Anim. Sci.* **25**(2):184-190.

Kaiser, B., Fareed, J., Hoppensteadt, D., Birdsong, B., Walenga, J.M. and Markwardt, F. 1992. Influence of recombinant hirudin and unfractionated heparin on thrombin and factor X_a generation in extrinsic and intrinsic activated systems. *Thromb. Res.* **65**:157-164.

Kaplan, A., Szabo, L.L. and Opheim, K.E. 1988. *Clinical Chemistry: Interpretations and Techniqu es.* 3d ed. Philadelphia: Lea and Febiger.

Kelly, A.B., Maraganore, J.M., Bourdon, P., hanson, S.R. and harker, L.A. 1992. Antithrombotic effects of synthtetic peptides targeting different functional domains of thrombin. *Proc. Natl. Acad. Sci. USA.* **89**:6040-6044.

Knapp, A., Degenhardt, T. and Dodt, J. 1992. Hirudisins: Hirudin-derived thrombin inhibitors with disintegrin activity. *Jour. Biol. Chem.* **267**(34):24230-24234.

Kokate, T.G., Svensson, B.J. and Rogawski, M.A. 1994. Anticonvulsant activity of neurosteroids: correlation with γ-aminobutyric acid-evoked chloride current potentiation. *Jour. Pharmacol. Exptl. Therapeut.* **270**(3):1223-1229.

Kozma, C., Macklin, W., Cummins, L.M. and Mauer, R. 1974. Anatomy, physiology, and biochemistry of the rabbit. In: *The Biology of the Laboratory Rabbit*, ed. Weisbroth, S.H., Flatt, R.E. and Kraus, A.L, 50-72. New York, San Francisco, London: Academic Press.

Kraus, A.L. 1980. Research Methodology. In: *The Laboratory Rat*, ed. H. J. Baker, J. R. Lindsey and S. H. Weisbroth, 1-42, vol. **II**. New York, London, Toronto, Sydney, San Francisco: Academic Press.

Kunno, S., Fenton II, J.W. and Villanueva, G.B. 1988. Analysis of the secondary structure of hirudin and the mechanism of its interaction with thrombin. *Arch. Biochem. Biophy.* **267**(1):158-166.

Lang, P.L. 1993a. Hematology parameters for the Crl:CD®BR rat. Charles River Laboratories, Inc., Wilmington, MA.

Lang, P.L. 1993b. Serum chemistry parameters for the Crl:CD®BR rat. Charles River Laboratories, Inc., Wilmington, MA.

Lazar, J.B., Winant, R.C. and Johnson, P.H. 1991. Hirudin: amino-terminal residues play a major role in the interaction with thrombin. *Jour. Biol. Chem.* **266**(2):685-688.

Lefkovits, J. and Topol, E. 1994. Direct thrombin inhibitors in cardiovascular medicine. *Circulation*. **90**:1522-1536.

Lent, C.M. and Dickinson, M.H. 1988. The neurobiology of feeding in leeches. *Sci. Amer.* 98-103.

Lindbratt, S., Eriksson, B.I., Ekman, S., Ahnfeldt, L. and Close, P. 1995. Less influence on liver enzymes with recombinant hirudin, CGP 39393, (^{TM}Revasc-Ciba), compared to unfractionated heparin, in patients undergoing total hip replacement. **Abstract.** To be presented at the XVth Congess of the *International Society on Thrombosis and Haemostasis*, Jerusalem, Israel, June 11-16, 1995.

Link, K.P. 1959. The discovery of dicumarol and its sequels. *Circulation.* **19**:97-107.

Loegering, D.J. 1974. Effect of swimming and treadmill exercise on plasma enzyme levels in rats. *Proc. Soc. Exptl. Biol. Med.* **147**:177-180.

Loison, G., Findeli, A., Bernard, S., Nguyen-Juilleret, M., Marquet, M., Reihl-Bellon, N., Carvallo, D., Guerra-Santos, L., Brown, S.W., Courtney, M., Roitsch, C. and Lemoine, Y. 1988. Expression and secretion in *Saccharomyces cerevisiae* of biologically active leech hirudin. *Bio/Tech.* **6**:72-77.

Luke, D.R., Wasan, K.M., Verani, R.R., Brunner, L.J., Berens, K.L., Vadiei, K. and Lopez-Berestein, G. 1991. Attenuation of amphotericin-B nephrotoxicity in the candidiasis rat model. *Nephron.* **59**:139-144.

Macfarlane, R.G. 1964. An enzyme cascade in the blood clotting mechanism, and its function as a biochemical amplifier. *Nature.* **202**:498-499.

Märki, W.E. and Wallis, R.B. 1990. The anticoagulant and antithrombotic properties of hirudins. *Thromb. Haemost.* **64**(3):344-348.

Markwardt, F. 1970. Hirudin as an inhibitor of thrombin. *Meth. Enzymol.* **19**:924-932.

Markwardt, F. 1988. Biochemistry and pharmacology of hirudin. In *Hematology: Hemostasis and Animal Venoms*. ed. H. Pirkle and F. S. Markland Jr. 255-269, vol. 7. New York, Basel: Marcel Dekker, Inc.

Markwardt, F. 1991a. Hirudin and derivatives as anticoagulant agents. *Thromb. Haemost.* **66**(1):141-152.

Markwardt, F. 1991b. Past, present and future of hirudin. *Haemostasis.* **21** (Suppl. 1):11-26.

Markwardt, F. 1994. The development of hirudin as an antithrombotic drug. *Thromb. Res.* **74**(1):1-23.

Markwardt, F. and Walsmann, P. 1958. Die reaktion zwischen hirudin und thrombin. *Hoppe-Seylers Z. Physiol. Chem.* **312**:85-98.

Markwardt, F. and Walsmann, P. 1967. Reindarstellung und analyse des thrombin inhibitors hirudin. *Hoppe-Seyler's Z. Physiol. Chem.* **348**:1381-1386.

Markwardt, F., Hauptmann, J., Nowak, G., Kleβen, Ch. and Walsmann, P. 1982. Pharmacological studies on the antithrombotic action of hirudin in experimental animals. *Thromb. Haemost.* **47**(3):226-229.

Markwardt, F., Nowak, G., Stürzebecher, J., Greiβbach, U., Walsmann, P. and Vogel, G. 1984. Pharmacokinetics and anticoagulant effect of hirudin in man. *Thromb. Haemost.* **52**(2):160-163.

Markwardt, F., Fink, G., Kaiser, B., Klocking, H.-P., Nowak, G., Richter, M. and Stürzebecher, J. 1988a. Pharmacological survey of recombinant hirudin. *Pharmazie.* **43**(3): 202-207.

Markwardt, F., Nowak, G., Stürzebecher, J. and Vogel, G. 1988b. Clinico-pharmacological studies with recombinant hirudin. *Thromb. Res.* **52**:393-400.

Markwardt, F., Kaiser, B. and Nowak, G. 1989. Studies on antithrombotic effects of recombinant hirudin. *Thromb. Res.* **54**:377-388.

Markwardt, F., Richter, M., Walsmann, P., Riesener, G. and Paintz, M. 1990. Preparation of dextran-bound recombinant hirudin and its pharmacokinetic behaviour. *Biomed. Biochim. Acta.* **10**:1103-1108.

Markwardt, F., Kaiser, B. and Richter, M. 1992. Haemostyptic effects of batroxobin with regard to hirudin treatment. *Thromb. Res.* **68**:475-482.

Marmo, E., Caputi, A.P. and Saini, R.K. 1973. cardiac histological and serum biochemical changes produced by various adrenergic stimulant drugs in the rat. *Basic Res. Cardiol.* **68**:318-334.

McLean, J. 1916. The thromboplastic action of cephalin. *Amer. Jour. Physiol.* **41**:250-57.

Meyer, B.H., Luus, H.G., Muller, F.O., Badenhorst, P.N. and Rothig, H.,-J. 1990. The pharmacology of recombinant hirudin, a new anticoagulant. *S. Afr. Med. Jour.* **78**:268-270.

Mille, B., Condamines, O., Herbert, J.M., Maftouh, M., Picard, C., Dussossoy, D. and Pau, B. 1994. Two-site immunoassay of recombinant hirudin based on two monoclonal antibodies. *Clin. Chem.* **40**(5):734-739, 1994.

Miller, I. and Freund, J.E. 1977. Curve fitting. In: *Probability and Statistics for Engineers*, ed. Miller, I. and Freund, J.E., 289-330, 2d ed., New Jersey: Prentice-Hall, Inc.

Mitruka, B.M. and Jonas, A.M. 1969. Biochemical changes in serum of pure and mixed *Streptococcus faecalis* and *Bacillus subtilis* infections in rats. *Appl. Microbiol.* **18**:1072-1076.

Murphy, J.E., Preuss, H.G. and Henry, J.B. 1984. Evaluation of renal function and water electrolyte, and acid-base balance. In: *Clinical Diagnosis and Management of Laboratory Methods*, ed. J.B. Henry, 118-132, 17th ed., Philadelphia, London, Toronto, Mexico City, Rio de Janeiro, Sydney, Tokyo: W.B. Saunders Company.

Naito, K. and Fujikawa, K. 1991. Activation of human blood coagulation factor XI independent of factor XII. *Jour. Biol. Chem.* **266**(12):7353-7358.

Nelson, D.A. and Henry, J.B. 1984. Hematology and coagulation. In: *Clinical Diagnosis and Management of Laboratory Methods*, ed. J.B. Henry, 578-765, 17th ed., Philadelphia, London, Toronto, Mexico City, Rio de Janeiro, Sydney, Tokyo: W.B. Saunders Company.

Nemerson, Y. and Repke, D. 1985. Tissue factor accelerates the activation of coagulation factor VII: The role of a bifunctional coagulation cofactor. *Thromb. Res.* **40**:351-358.

Neuhaus, K.-L., Essen, R.V., Tebbe, U., Jessel, A., Heinrichs, H., Mäurer, Döring, W., Harmjanz, D., Kötter, V., Kahlhammer, E., Simon, H. and Horacek, T. 1994. Safety observations from the pilot phase of the randomized r-hirudin for improvement of thrombolysis (HIT-III) study. *Circulation*. **90**:1638-1642.

Nowak, G. and Bucha, E. 1994. Ecarin-induced prothrombin intermediate formation: an effective antidate principle to antagonoze toxic levels of hirudin. *Proceedings of the Fifth Annual Symposium on Advances in Anticoagulant, Antithrombotic and Thrombolytic Therapeutics, in Boston, October 24-25, 1994,* by International Business Communications.

Nowak, G., Markwardt, F. and Fink, E. 1988. Pharmacokinetic studies with recombinant hirudin in dogs. *Folia Haematol.* **115**(1-2, Suppl):70-74.

Ofosu, F.A., Blajchman, M.A., Modi, G., Cerskus, A. and Hirsh, J. 1981. Activation of factor X and prothrombin in antithrombin III depleted plasma: the effects of heparin. *Thromb. Res.* **23**:331-345.

Ofosu, F.A., Modi, G., Cerskus, A.L., Hirsh, J. and Blajchman, M.A. 1982. Heparin with low affinity to antithrombin III inhibits the activation of prothrombin in normal plasma. *Thromb. Res.* **28**:487-497.

Ofosu, F.A., Fernandez, F., Gauthier, D. and Buchanan, M.R. 1985. Heparin cofactor II and other endogenous factors in the mediation of the antithrombotic and anticoagulant effects of heparin and dermatan sulfate. *Sem. Thromb. Hemost.* **11**(2):133-137.

Pearson, J.D. 1994. Endothelial cell biology. In: *Haemostasis and Thrombosis,* ed. A. L. Bloom, C. D. Forbes, D. P. Thomas and G. D. Tuddenham, 219-232, 3d ed., vol. I. Edinburgh, London, Madrid, Melbourne, New York, Tokyo: Churchill Livingstone.

Petersen, T.E., Roberts, H.R., Sottrup-Jensen, L. and Magnusson, S. 1976. Primary structure of hirudin, a thrombin-specific inhibitor. In *Protides of the Biological Fluids*. ed. H. Peeters. 145-149, proc. 23rd colloquium. Oxford, New York: Pergamon Press.

Plachetka, J.R. 1994. Overview of argatroban. In *Proceedings of the Fifth Annual Symposium on Advances in Anticoagulant, Antithrombotic and Thrombolytic Therapeutics, in Boston, October 24-25, 1994,* by International Business Communications.

Pizzo, S.V., Friedberg, R.C., Szw, P., Winant, R., Hidson, D. and Lazar, J.M. 1990. Recombinant hirudin displaces human factor X_a from its enothelial binding sites. *Thromb. Res.* **57**(5):803-806.

Poller L. 1980. Standardization of the APTT test. Current status. *Scand. Jour. Haematol.* **37** (suppl.):49-63.

Porta, R., Pescador, R., Mantovani, M. and Prino, G. 1990. Quantitative comparison of recombinant hirudin's antithrombotic and anticoagulant activities with those of heparin. *Thromb. Res.* **57**:639-643.

Powers, J.C. and Kam, C. 1992. Synthetic substrates and inhibitors of thrombin. In *Thrombin: Structure and Function*, ed. L.J. Berliner, New York:Plenum Press.

Prescott, L.F., Freestone, S. and McAuslane, J.A.N. 1991. Reassessment of the single intravenous injection method with inulin for measurement of the glomerular filtration rate in man. *Clin. Sci.* **80**:167-176.

Raake, W., Klauser, R.J., Elling, H. and Zeiller, P. 1991. Antithrombotic action of recombinant hirudin in a venous thrombosis model. *Haemostasis.* **21** (Suppl. 1):127-132.

Racanelli, A. 1990. Biochemical and pharmacological studies on the interaction of protamine with heparins. Ph.D. diss., Loyola University Chicago.

Ratnoff, O.D. 1965. The blood clotting mechanism and its disorders. *Disease-a-month*. 1-49.

Ratnoff, O.D. 1974. Some recent advances in the study of hemostasis. *Cir. Res.* **35**(1):1-14.

Ratnoff, O.D. 1994. The development of knowledge about haemostasis and thrombosis. In: *Haemostasis and Thrombosis*, ed. A. L. Bloom, C. D. Forbes, D. P. Thomas and G. D. Tuddenham, 3-28, 3d ed., vol. I. Edinburgh, London, Madrid, Melbourne, New York, Tokyo: Churchill Livingstone.

Rescigno, A. and Beck, J.S. 1987. The use and abuse of models. *Jour. Pharmacokin. Biopharmaceut.* **15**(3): 327-341.

Richter, M., Cyranka, U., Nowak, G. and Walsmann, P. 1988. Pharmacokinetics of ^{125}I-hirudin in rats and dogs. *Folia Haematol.* **115**(1-2, S.):64-69.

Richter, M., Walsmann, P. and Markwardt, F. 1989. Plasma level of dextran-r-hirudin. *Pharmazie*. **44**(H.1):73-74.

Rieger, M., Fortkamp, E., Schweitzer, S., Heisterberg, M. and Sommer, R. 1988. Studies on the expression of a synthetic hirudin gene in *Escherichia coli*. In *Hematology: Hemostasis and Animal Venoms*. ed. H. Pirkle and F. S. Markland Jr. 295-306, vol. **7**. New York, Basel: Marcel Dekker, Inc.

Riehl-Bellon, N., Carvallo, D., Acker, M., Van Dorsselaer, A., Marquet, M., Loison, G., Lemoine, Y., Brown, S.W., Courtney, M. and Roitsch, C. 1989. Purification and biochemical characterization of recombinant hirudin produced by *Saccharomyces cerevisiae*. *Biochemistry*. **28**:2941-2949.

Ringler, D.H. and Dabich, L. 1979. Hematology and clinical biochemistry. In: *The Laboratory Rat*, ed. H. J. Baker, J. R. Lindsey and S. H. Weisbroth, 105-121, vol. **I**. New York, London, Toronto, Sydney, San Francisco: Academic Press.

Roberts, H.R. and Lozier, J.N. 1992. New perspectives on the coagulation cascade. *Hosp. Practice*. **27**(1):97-112.

Rohrer, M.J. and Natale, A.M. 1992. Effect of hypothermia on the coagulation cascade. *Crit. Care Med*. **20**(10):1402-1405.

Romisch, J., Stohr, H-A., Stauß, H., Koschinsky, R., Stuber, W. and P, E-P. 1994. Inhibition of in vitro clot growth by r-hirudin is more effective and longer sustained than by an analogous peptide. *Thromb. Haemost*. **71**(3):320-324.

Rowland, M. and Tozer, T.N. 1989. *Clinical Pharmacokinetics: Concepts and Applications*. 2d ed. Philadelphia, London: Lea and Febiger.

Rübsamen, K. 1994. Chemical derivatisation of hirudin as a strategy to meet different therapeutic requirements. In *Proceedings of the Fifth Annual Symposium on Advances in Anticoagulant, Antithrombotic and Thrombolytic Therapeutics, in Boston, October 24-25, 1994*, by International Business Communications.

Rydel, T.J., Ravichandran, K.G., Tulinsky, A., Bode, W., Huber, R., Roitsch, C. and Fenton II, J.W. 1990. The structure of a complex of recombinant hirudin and human α-thrombin. *Science*. **249**:277-280.

Rydel, T.J., Tulinsky, A., Bode, W. and Huber, R. 1991. Refined structure of the hirudin-thrombin complex. *Jour. Mol. Biol.* **221**:583-601.

Saito, H. 1994. The contact phase of blood coagulation. In: *Haemostasis and Thrombosis*, ed. A. L. Bloom, C. D. Forbes, D. P. Thomas and G. D. Tuddenham, 289-307, 3d ed., vol. I. Edinburgh, London, Madrid, Melbourne, New York, Tokyo: Churchill Livingstone.

Santoro, F.M., Alvarez, R. and Fussi, F. 1992. Pharmacological profile of a native dermatan sulfate. *Thromb. Res.* **67**:201-211.

Sarembock, I.J., Gertz, D., Gimple, L.W., Owen, R.M., Powers, E. and Roberts, W.C. 1991. Effectiveness of recombinant desulfatohirudin in reducing restenosis after balloon angioplasty of atherosclerotic femoral arteries in rabbits. *Circulation*. **84**(1):232-243.

Sawyer, R.T. 1988. Scientific rationale behind the medicinal use of leeches. In *Hematology: Hemostasis and Animal Venoms*. ed. H. Pirkle and F. S. Markland Jr. 271-279, vol. 7. New York, Basel: Marcel Dekker, Inc.

Schalm, O.W., Jain, N.C. and Carroll, E.J. 1975. *Veterinary Hematology*. 3d ed., Philadelphia: Lea and Febiger.

Scharf, M., Engels, J. and Tripier, D. 1989. Primary structures of new 'iso-hirudins'. *FEBS Letters*. **255**(1):105-110.

Seemuller, U., Dodt, J., Fink, E. and Fritz, H. 1986. Proteinase Inhibitors of the Leech *Hirudo medicinalis* (hirudins, bdellins, eglins). In *Proteinase Inhibitors*. ed. A. J. Barrett and G. Salvesen, 337-359. Amsterdam, New York, Oxford: Elsevier.

Sharma, G.V.R.K., Lapsley, D., Vita, J., Sharma, S., Coccio, E., Adelman, B. and Loscalzo, J. 1993. Usefulness and tolerability of *Hirulog*, a direct-thrombin inhibitor, in unstable angina pectoris. *Amer. Jour. Cardiol.* **72**:1357-1360.

Spannagl, M., Bichler, H., Lill, H. and Schramm, W. 1991. A fast photometric assay for the determination of hirudin. *Haemostasis*. **21**(Suppl.1):36-40.

Spinner, S., Stoffler, G. and Fink, E. 1986. Quantitative enzyme-linked immunosorbent assay (ELISA) for hirudin. *Jour. Immunol. Meth.* **87**:79-83.

Spinner, S., Scheffauer, F., Maschler, R. and Stoffler, G. 1988. A hirudin catching ELISA for quantitating the anticoagulant in biological fluids. *Thromb. Res.* **51**(6):617-25.

Stocker , K. 1991. Laboratory use of hirudin. *Sem. Thromb. Haemost.* **17**(2):113-121.

Stone, S.R. and Hofsteenge, J. 1987. Effect of heparin on the interaction between thrombin and hirudin. *Eur. Jour. Biochem.* **169**:373-376.

Stone, S.R. and Maraganore, J. 1992. Hirudin ineractions with thrombin. In *Thrombin. Structure and Function*. ed. L. J. Berliner, 219-256. New York, Plenum Press.

Stuever, T.A., Fareed, J., Hoppensteadt, D., Callas, D. and Iqbal, O. 1995. Neutralization of recombinant hirudin by FEIBA®. *In Preparation*.

Topol, E.J., Bonan, R., Jewitt, D., Sigwart, U., Kakkar, V.V., Rothman, M., de Bono, D., Ferguson, J., Willerson, J.T., Strony, J., Ganz, P., Cohen, M.D., Raymond, R., Fox, I., Maraganore, J.M. and Adelman, B. 1993. Use of a direct antithrombin, hirulog, in place of heparin during coronary angioplasty. *Circulation.* **87**:1622-1629.

Topol, E.J., Fuster, V., Harrington, R.A., Califf, R.M., Kleiman, N.S., Kereiakes, D.J., Cohen, M., Chapekis, A., Gold, H.K., Tannenbaum, M.A., Rao, A.K., Debowey, D., Schwartz, D., Henis, M. and Chesebro, J. 1994. Recombinant hirudin for unstable angina pectoris. A multicenter, randomized angiographic trial. *Circulation*. **89**(4):1557-1566.

Tripodi, A, Chantarangkul, V., Arbini, A.A., Moia, M. and Mannucci, P.M. 1993. Effects of hirudin on activated partial thromboplastin time determined with ten different reagents. *Thromb. Haemost*. **70**(2):286-288.

Tuong, A., Maftouh, M., Ponthus, C., Whitechurch, O., Roitsch, C. and Picard, C. 1994. Characterization of the deamidated forms of recombinant hirudin. *Biochemistry*. **31**(2):8291-8299.

Vadiei, K., Berens, K.L. and Luke, D.R. 1990. Isolation-induced renal functional changes in rats from four breeders. *Lab. Anim. Sci.* **40**:56-59.

Valentin, S., Ostergaard, P., Kristensen, H. and Nordfang, O. 1992. Synergism between full length TFPI and heparins: evidence for TFPI as an importent factor for the antithrombotic activity of heparin. *Blood Coag. Fibrinol.* **3**(2):221-222.

Van Dorsselaer, A., Lepage, P., Bitsch, F., Whitechurch, O., Riehl-Bellon, N., Fraisse, D., Green, B. and Roitsch, C. 1989. Mass spectrometry analyses of recombinant hirudins (7 kDa). *Biochemistry*. **28**:2949-2956.

Verstraete, M., Nurmohamed, M., Kienast, J., Siebeck, M., Silling-Englehardt, G., Buller, H., Hoet, B., Bichler, J. and Close, P., on behalf of the European Hirudin in Thrombosis Group. 1993. Biologic effects of recombinant hirudin (CGP 39393) in human volunteers. *Jour. Amer. Coll. Cardiol.* **22**:1080-1088.

Vogel, G. and Markwardt, F. 1988. Clinical use of hirudin. *Folia Hematol. Leipz*. **115**(1-2,S.):113-118.

Waddell, W.J. 1956. A simple ultraviolet spectrophotometric method for the determination of protein. *Jour. Lab. Clin. Med.* **48**:311-314.

Wagner, 1975. Do you need a pharmacokinetic model, and, if so, which one? *Jour. Pharmacokin. Biopharmaceut.* **3**(6):478.

Walenga, J.M., 1987. Factor X_a inhibition in mediating antithrombotic actions: application of a synthetic heparin pentasaccharide. Ph.D. diss., University of Paris.

Walenga, J.M., Pifarre, R. and Fareed, J. 1990. Recombinant hirudin as an antithrombotic agent. *Drugs Future*. **15**(3):267-280.

Walenga, J.M., Hoppensteadt, D., Koza, M., Wallock, M., Pifarre, R. and Fareed, J. 1991a. Laboratory assays for the evaluation of recombinant hirudin. *Haemostasis.* **21**(suppl. 1):49-63.

Walenga, J.M., Mamdouh, B., Messmore, H.L., Fareed, J. and Pifarre, R. 1991b. Potential use of recombinant hirudin as an anticoagulant in a cardiopulmonary bypass model. *Ann. Thorac. Surg*. **51**:271-277.

Walenga, J.M., Markwardt, F., Breddin, K. and Fareed, J. 1991c. Report on a discussion forum: "Medicinal and surgical application of recombinant hirudin". *Sem. Thromb. Hemost*. **17**(2):150-156.

Walker, F.J. 1981. Regulation of activated protein C by protein S. The role of phospholipid in factor Va inactivation. *Jour. Biol. Chem.* **256**:11128-11131.

Walsmann, P. and Markwardt, F. 1985. On the isolation of the thrombin inhibitor hirudin. *Thromb. Res.* **40**:563-569.

Weichart, W., Pauliks, V. and Breddin, H.K. 1983. Laser-induced thrombosis in rat mesenteric vessels and antithrombotic drugs. *Haemostasis.* **13**:61-171.

Weitz, J.I., Hudoba, M. and Massel, D. 1990. Clot-bound thrombin is protected from inhibiton by heparin-antithrombin-III but is susceptible to inactivation by antithrombin-III independent inhibitors. *Jour. Clin. Invest.* **86**:385-391.

Wessler, S., Reimer, S.M. and Sheps, M.C. 1959. Biological assay of a thrombosis-inducing activity in human serum. *Jour. Appl. Physiol.* **14**(6):943-946.

West, B.R., Nichter, L.S. and Halpern, D.E. 1994. Emergent reuse in leech therapy: a better method. *Plast. Reconst. Surg.* **93**(5):1095-1098.

White, H. 1993. Hirudin and hirulog. *Aust. NZ. Med Jour.* **23**:769-770.

Wilkinson, L. 1990. SYSTAT: The system for statistics. Evanston, IL: SYSTAT, Inc.

Winant, R.C., Lazar, J.B. and Johnson, P.H. 1991. Chemical modifications and amino acid substitutions in recombinant hirudin that increase hirudin-thrombin affinity. *Biochemistry.* **30**(5):1271-1277.

Witting, J.I., Bourdon, P., Brezniak, D.V., Maraganore, J.M. and Fenton II, J.W. 1992. Thrombin-specific inhibition by and slow cleavage of hirulog-I. *Biochem. Jour.* **283**:737-743.

Yamaoka, K., Nakagawa, T. and Uno, T. 1978. Statistical moments in pharmacokinetics. *Jour. Pharmacokin. Biopharm.* **6**(6):547-558.

Yates, F.E. 1978. Good manners in good modeling: mathematical models and computer simulations of physiological systems. *Amer. Jour. Physiol.* **3**:R159-R160.

Zar, J.H. 1984. *Biostatistical Analysis*. 2d ed. New Jersey: Prentice-Hall, Inc.

Zimmerman, H.J. 1984. Function and integrity of the liver. In: *Clinical Diagnosis and Management of Laboratory Methods*, ed. J.B. Henry, 217-250, 17th ed., Philadelphia, London, Toronto, Mexico City, Rio de Janeiro, Sydney, Tokyo: W.B. Saunders Company.

Zitoun, D., Bara, L., Bloch, M.F. and Samama, M.M. 1994. Plasma TFPI activity after intravenous injection of pentasaccharide (PS) and unfractionated heparin in rabbits. *Thromb. Res.* **75**(5):577-580.

VITA

The author, Lalitha Iyer, was born on September 13, 1964, in Bombay, India. She received her higher secondary education at The Ramnarain Ruia College, Bombay, and obtained a Bachelor of Pharmacy degree from The Bombay College of Pharmacy, University of Bombay, in 1985.

Ms. Iyer enrolled in the graduate program in Pharmaceutics at The College of Pharmacy, University of Houston, under the direction of Dr. David Luke, in 1988. Along with her husband, Dr. Shankar Krishnan, Ms. Iyer moved to Chicago in 1989, and continued her graduate work in the Department of Pharmacology at Loyola University Chicago, in July, 1989, with a basic science fellowship. She joined the laboratory of Dr. Jawed Fareed to pursue research in vascular pharmacology. Ms. Iyer has presented her research work and participated actively in several national and international conferences. Ms. Iyer was awarded the Arthur J. Schmitt Fellowship in 1993, and is a member of Alpha Sigma Nu, the Jesuit Honor Society. Ms. Iyer is a student member of the American Association of Pharmaceutical Scientists (AAPS), the International Society on Thrombosis and Haemostasis (ISTH), and the American Association of Clinical Chemistry (AACC).

Ms. Iyer has been awarded a postdoctoral fellowship by the Committee on Clinical Pharmacology, at the Pritzker School of Medicine, University of Chicago.

Selected Publications

Iyer, L. and Fareed, J. 1995. Recombinant hirudin: a perspective. Submitted to *Clin. App. Thromb. Haemost.*

Iyer, L. and Fareed, J. 1995. Recombinant hirudin does not compromise renal function in rats. Submitted to *Drug Metab. Dispos.*

Iyer, L. and Fareed, J. 1995. Determination of specific activity of recombinant hirudin using a thrombin titration method. *Thromb. Res.* In press.

Iyer, L., Adam, M., Amiral, J., Fareed, J. and Bermes, E.W., Jr. 1995. Development and validation of an enzyme-linked immunosorbent assay (ELISA) to measure recombinant hirudin in biological fluids. *Sem. Thromb. Haemost.* In press.

Iyer, L., Koza, M., Iqbal, O., Calabria, R. and Fareed, J. 1993. Studies on the pharmacokinetics and pharmacodynamics of recombinant hirudin (rHV2-Lys 47) after intravenous and subcutaneous administration in dogs. *Thromb. Res.* 69:259-269.

Iyer, L., Shavit, J., Koza, M., Calabria, R., Moran, S. and Fareed, J. 1993. Alteration of pharmacokinetics and pharmacodynamics of recombinant hirudin (rHV2-Lys 47) after repeated intravenous administration in dogs. *Thromb. Res.* 69:59-70.

Fareed, J., Walenga, J.M., Iyer, L., Hoppensteadt, D. and Pifarre, R. 1991. An objective perspective on recombinant hirudin: a new anticoagulant and antithrombotic agent. *Blood Coag. Fibrinol.* 2:135-147.

Selected Abstracts

Iyer, L., Iqbal, O. and Fareed, J. 1995. Relevance of pharmacokinetics with pharmacodynamics of recombinant hirudin in dogs. *Experimental Biology '95, Atlanta, GA.* Accepted for presentation.

Iyer, L., Asselmeier, S., Iqbal, O. and Fareed, J. 1994. Evaluation of renal function and dispositional characteristics of recombinant hirudin in rats. *The FASEB Jour.* 8(4):A98.

Iyer, L., Fareed, J., Ovsepian, A. and Amiral, J. 1993. Time-course of antithrombotic action of recombinant hirudin variant 2 (rHV2-Lys 47) after intravenous and subcutaneous administration in rabbits. *The FASEB Jour.*, 7(3):A483.

Iyer, L., Fareed, J., Amiral, J. and Adam, M. 1993. Application of a competitive enzyme-linked immunosorbent assay method in the study of pharmacokinetics of recombinant hirudin in animal models. *Thromb. Haemost.* 69(6):1304.

Iyer, L., Amiral, J., Fareed, J. and Bermes, E.W. Jr. 1993. Evidence for a matrix effect on the recovery of recombinant hirudin using a competitive enzyme-linked immunosorbent assay (ELISA) method. *Clin. Biochem. Rev.* 14(iv):212.

Iyer, L., Khenkina, Y., Amiral, J. and Fareed, J. 1992. A profile of antithrombotic and bleeding effects of recombinant hirudin variant 2 (rHV2-Lys 47). *Proceedings of the 3rd annual meeting on Advances in Anticoagulant, Antithrombotic and Thrombolytic agents, International Business Communications, Boston, MA.*

Iyer, L., Koza, M., Iqbal, O., Calabria, R. and Fareed, J. 1992. Influence of route of administration on the pharmacokinetics and pharmacodynamics of recombinant hirudin. *The Pharmacologist.* 34(3):181.

Iyer, L., Hoppensteadt, D., Walenga, J.M. and Fareed, J. 1990. Development and application of methods for monitoring the pharmacokinetics/ pharmacodynamics of recombinant hirudins and related agents. *The Pharmacologist.* 32(3):185.

Selected Oral Presentations

Iyer, L. 1993. Pharmacokinetic and pharmacodynamic analysis of recombinant hirudin variant 2 in animal models. Central Haematological Laboratory, Hotel Dieu at the University of Paris, France.

Iyer, L. 1992. Pharmacological profiling of recombinant hirudin in valid models. International Biotechnology Expo and Scientific Conference (IBEX, '92), San Francisco, CA.

Iyer, L., Koza, M., Iqbal, O., Shavit, J. and Fareed. J. 1991. Pharmacokinetics and pharmacodynamics of recombinant hirudins. *The FASEB Jour.* 5(5):A1204.

DISSERTATION APPROVAL SHEET

The dissertation submitted by Lalitha Iyer has been read and approved by the following committee:

Jawed Fareed, Ph.D. (Director),
Professor of Pathology and Pharmacology,
Director, Hemostasis Research Laboratories,
Loyola University Medical Center.

Edward, W. Bermes, Jr., Ph.D.,
Professor and Associate Chairman of Pathology,
Director, Clinical Laboratory Division,
Loyola University Medical Center.

Israel Hanin, Ph.D.,
Professor and Chairman,
Department of Pharmacology,
Director, Neuroscience and Aging Institute,
Loyola University Medical Center.

Stanley Lorens, Ph.D.,
Professor of Pharmacology,
Loyola University Medical Center.

David Luke, Pharm.D.,
Associate Director,
Clinical Research,
Pfizer, Inc.,
Groton, CT 06340.

Harry L. Messmore, M.D.,
Professor of Medicine and Pathology,
Stritch School of Medicine,
Loyola University Cancer Center.

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.

Apr. 7, 1995
Date

Director's signature