A STUDY OF THE RELATIONSHIPS BETWEEN THE PHARMACOKINETICS AND THE PHARMACODYNAMICS OF WARFARIN AND VITAMIN K_1

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

by

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To my Mum and Dad, Mavis and Frank Cholerton

ABSTRACT

An animal model has been developed in which the pharmacokinetics and the pharmacodynamics of warfarin and its enantiomers have been investigated and compared. Using mathematical models, which have been shown to be suitable for the investigation of warfarin over a ten-fold dose range, the relative contributions of the pharmacokinetics and the pharmacodynamics to the overall pharmacological effect of this coumarin anticoagulant and its enantiomers were determined, after single intravenous doses, in the rabbit.

This method of pharmacological analysis, has been employed to distinguish between the pharmacokinetics and the pharmacodynamics of warfarin in patients before and after effective treatment of thyrotoxicosis. There was no significant difference in the pharmacokinetics of a single oral dose of warfarin between the thyrotoxic and euthyroid state. However, plasma concentration of warfarin-response (% of maximum rate of clotting factor synthesis) curves illustrated an apparent increase in receptor sensitivity to warfarin in the hypermetabolic state. This has been attributed to increased protein (clotting factor) turnover.

An investigation of the interaction between warfarin and cimetidine revealed a significant decrease in the plasma clearance of R(+) warfarin but no change in that for S(-) warfarin. This stereoselective interaction has been attributed to regioselective inhibition of 6-hydroxylation of warfarin.

Plasma concentrations of racemic and S(-) warfarin were shown to be no better than daily dose of warfarin as predictors of the pharmacological effect of warfarin. Furthermore, the accumulation of vitamin K_1 2,3-epoxide in plasma following a pharmacological dose of vitamin K_1, proved to be a poor quantitative marker of warfarin effect in patients. Nevertheless, plasma concentration of vitamin K_1 2,3-epoxide has been shown to be a useful qualitative marker of the pharmacological effect of warfarin in controlled volunteer studies. The production of plasma warfarin concentration-response (accumulation of vitamin K_1 2,3-epoxide) curves demonstrated a greater sensitivity of plasma vitamin K_1 2,3-epoxide when compared to the prothrombin time assay. This illustrated its usefulness in the detection of industrial exposure to brodifacoum and difenacoum.

The potent coumarin anticoagulant, brodifacoum, has been shown to have the ability to deplete both physiological plasma and hepatic concentrations of vitamin K_1, but to have no effect on the β-phase and the previously undefined γ-phase of vitamin K_1 elimination in the rabbit. A lack of simple equilibration between hepatic and plasma concentrations of vitamin K_1 following a pharmacological dose of the vitamin has been demonstrated. Thus, the rat was developed as an animal model with which to investigate hepatic concentration of vitamin K_1 in relation to pharmacological effect. The hepatic concentration of vitamin K_1 required to drive clotting factor synthesis in the anticoagulated rat was shown to be approximately 100 times greater than that present under normal circumstances.

CONTENTS

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The following papers, arising from the work presented in this thesis, have been published:-

1. BRECKENRIDGE, A.M., CHOLERTON, S., HART, J.A.D., PARK, B.K. and SCOTT, A.K. (1985).
   A study of the relationship between the pharmacokinetics and the pharmacodynamics of the 4-hydroxycoumarin anticoagulants warfarin, difenacoum and brodifacoum in the rabbit.
   Br. J. Pharmac., 84, 81-91.

2. CHOONARA, I.A., SCOTT, A.K., HAYNES, B.P., CHOLERTON, S., BRECKENRIDGE, A.M. and PARK, B.K. (1985).
   Vitamin K_1 metabolism in relation to pharmacodynamic response in anticoagulated patients.
   Br. J. clin. Pharmac., 20, 643-648.

3. CHOONARA, I.A., CHOLERTON, S., HAYNES, B.P., BRECKENRIDGE, A.M. and PARK, B.K. (1986).
   Stereoselective interaction between the R enantiomer of warfarin and cimetidine.
   Br. J. clin. Pharmac., 21, 271-277.

4. CHOLERTON, S. and PARK, B.K. (1986).
   Quantitative analysis of pharmacological concentrations of vitamin K_1 and vitamin K_1 2,3-epoxide in rat liver by high performance liquid chromatography.
   J. Chromatogr., 375, 147-153.

5. KELLETT, H.A., SAWERS, J.S.A., BOULTON, F.C., CHOLERTON, S., PARK, B.K., and TOFT, A.D. (1986).
Problems of anticoagulation with warfarin in hyperthyroidism.
Q.J. Med., 58, 43-51.

CHEMICAL NAMES

Vitamin K_1	2-methyl-3-phytyl-1,4-naphthoquinone.
Vitamin K_1 2,3-epoxide	2-methyl-3-phytyl-1,4-naphthoquinone-2,3-epoxide.
2-chlorovitamin K (Cl-K)	2-chloro-3-phytyl-1,4-naphthoquinone.
MK4	2-methyl-3-geranyl-geranyl-1,4-naphthoquinone.
MK6	2-methyl-3-farnesyl-farnesyl-1,4-naphthoquinone.
Warfarin	3-(α-acetonylbenzyl)-4-hydroxycoumarin.
Brodifacoum	3-(3-[4'bromo(1-1'-biphenyl)]-4-yl-1,2,3,4-tetrahydro-1-naphthalenyl)-4-hydroxy-2H-1-benzopyran-2-one.

ABBREVIATIONS

AUC	area under the plasma concentration-time curve
a.u.f.s.	absorbance units full scale
cm	centimetre
^{o}C	degree centigrade
Cp_{max}	maximum plasma concentration
Glu	glutamic acid residue
Gla	γ-carboxyglutamic acid residue
GLC	gas liquid chromatography
g	gram
g	gravitational force
HPLC	high performance liquid chromatography
h	hour
i.d.	internal diameter
i.p.	intraperitoneal
i u	international unit
kg	kilogram
l	litre
M	molar

mCi	millicurie
mg	milligram
min	minute
ml	millilitre
mm	millimetre
mmol	millimole
ng	nanogram
nm	nanometre
P.C.A.	prothrombin complex activity
pg	picogram
psi	pounds per square inch
PTR	prothrombin ratio
PTT	partial thromboplastin time
(R)	registered trade mark
r.p.m.	revolutions per minute
s	second
TLC	thin layer chromatography
UV	ultraviolet
µCi	microcurie

µg	microgram
µl	microlitre
v	volt
vol	volume
v/v	volume by volume
w/v	weight by volume

CHAPTER 1

INTRODUCTION

1.1 The History of Vitamin K and 4-hydroxycoumarin Anticoagulants

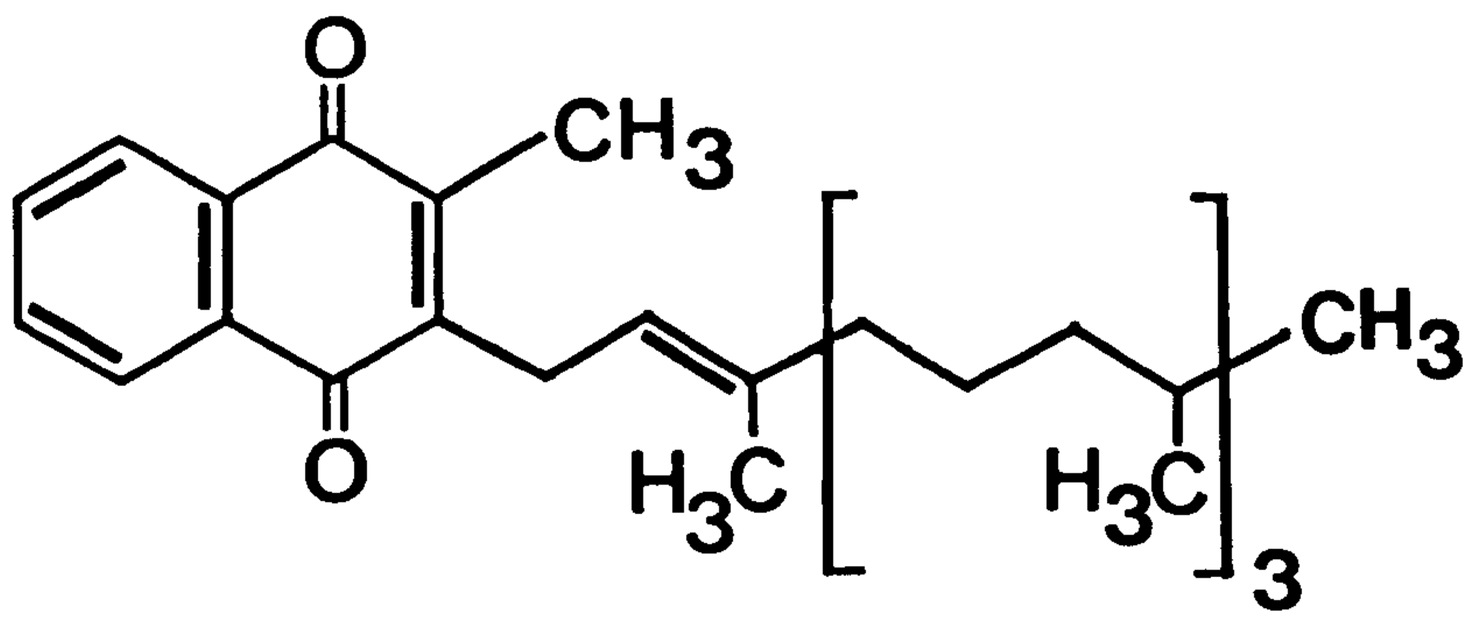
Warfarin is the mainstay of oral anticoagulant therapy in this country. It exerts its pharmacological effect by antagonism of vitamin K, the essential co-factor for clotting factor synthesis. By a remarkable coincidence, the discovery of these two compounds occurred almost simultaneously but independently in the 1920s and 1930s.

In 1929, Dam described the occurrence of a bleeding condition in chicks maintained on a diet deficient in ether soluble components. Two independent groups determined that the haemorrhagic disease could be prevented by the addition of certain extracts to the diet (Almquist and Stokstad, 1935; Dam, 1935). These workers determined that the preventative factor was present in the fat soluble fraction of a concentrated alfalfa meal extract, but was not identical to any of the known fat soluble vitamins. However, Dam was the first to report the existence of the "Koagulations Vitamin" as he described vitamin K. At the same time, Schonheyder (1935) discovered that the vitamin controlled prothrombin concentrations in blood, but that the vitamin itself had no prothrombin like activity.

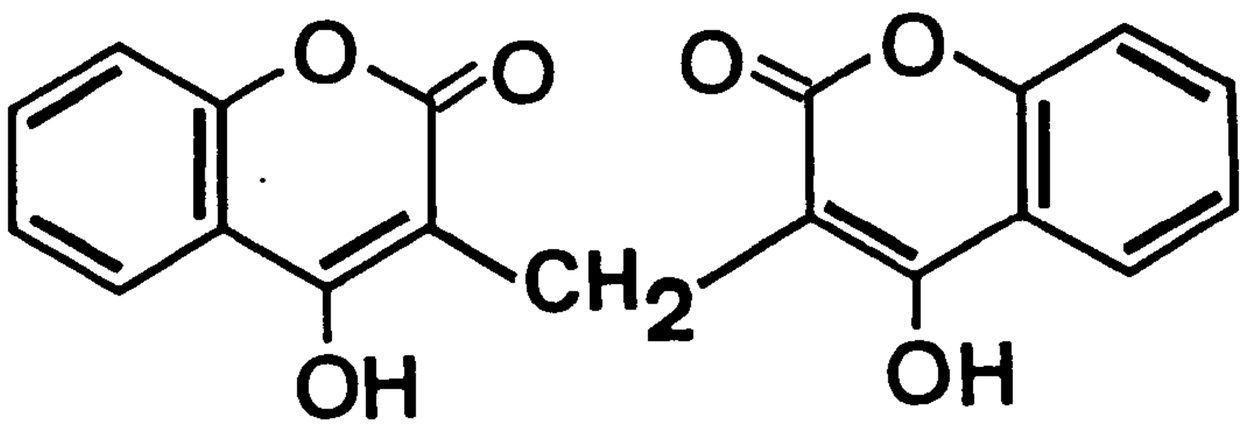
Vitamin K was found in green leafy plants especially alfalfa (Almquist, 1975), bacteria (McKee et al., 1939) and in the faeces of vitamin K deficient chicks (Clark et al., 1939). In 1939, vitamin K, (Figure 1.1) was isolated, identified and synthesised in four different laboratories (Almquist and Klose 1939; Binkley et al., 1939; Dam et al., 1939; Fieser, 1939). In 1944, Dam and Doisey shared the Nobel Prize for their contribution to this work.

During the early 1920s throughout the USA and Canada, an often fatal haemorrhagic disease of cattle existed. A veterinary surgeon

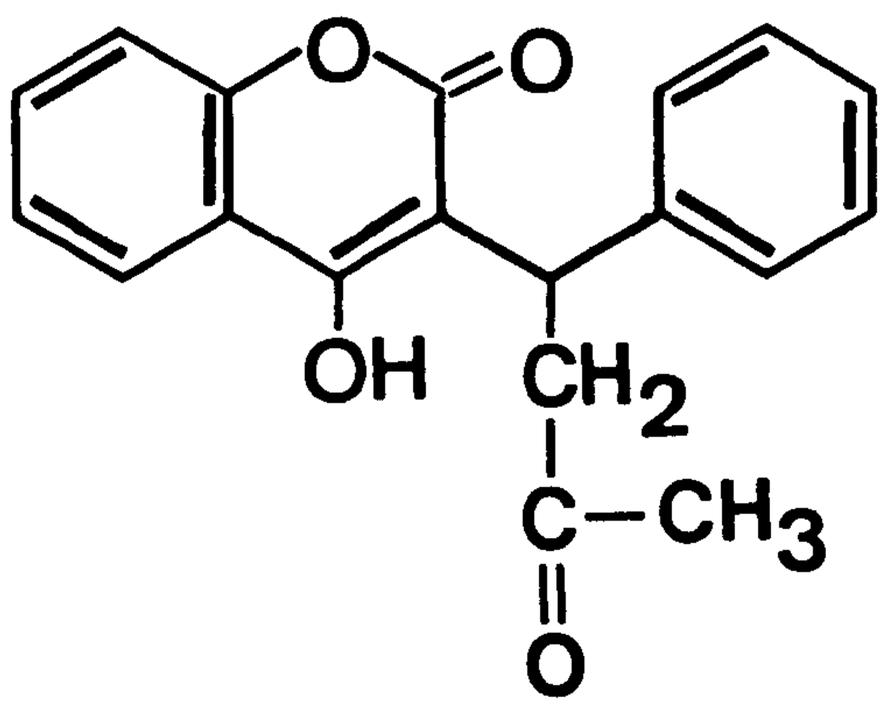
FIGURE 1.1 Chemical structures of vitamin K_1, dicoumarol and warfarin.



Vitamin K_1



Dicoumarol



Warfarin

named Schofield (1922) reported that the bleeding disorder was due to the ingestion of spoiled sweet clover hay. By stopping the clover feeds, and transfusing blood from normal cattle, the condition was seen to be reversed (Schofield, 1924). Some years later, an extremely low prothrombin concentration was demonstrated in animals suffering from this "Sweet Clover Disease" (Roderick, 1931).

By 1941, Link and co-workers had isolated the agent in spoiled sweet clover responsible for the bleeding disorder. This was dicoumarol (3, 3' - methylenebis 4-hydroxycoumarin; Figure 1.1) and its hypoprothrombinaemic effect was shown to be successfully alleviated by the administration of a large dose of vitamin K extracted from alfalfa (Link, 1945). Thus dicoumarol was shown to be a vitamin K antagonist.

On this evidence, dicoumarol was hailed a likely antithrombotic agent and by 1941, the first clinical trials had begun (Bingham et al.). The results were promising, and it was suggested that dicoumarol could take over from heparin in the treatment of thrombo-embolic disorders. This prompted a great deal of research on a large number of dicoumarol derivatives (Lehmann, 1943; Overman et al., 1945).

One such compound was warfarin (Figure 1.1), and in 1948 Link suggested its use as a rodenticide, since it had been demonstrated to be a particularly effective anticoagulant in the rat. Warfarin quickly became the most successful rodenticide ever produced. However, by the early 1960s the existence of warfarin resistant rats questioned its continued use.

The first reported case of human ingestion of warfarin was in a suicide attempt in 1952 (Holmes and Love). Nevertheless, by the mid 1950s warfarin had superseded dicoumarol in oral anticoagulant therapy,

and has continued to be the most popular coumarin anticoagulant in this country up to the present time.

Throughout the years of clinical use of warfarin, many improvements have been made regarding treatment with this compound. Nevertheless, anticoagulant therapy with warfarin is still far from perfect.

To improve further the clinical use of warfarin, it is necessary to consider the pharmacology of vitamin K_1 and warfarin, and also to determine the effect of these two compounds on the blood coagulation mechanisms.

1.2 The Pharmacology of Vitamin K

The K group of vitamins is comprised of two major chemical forms, both fat soluble and both widely distributed in nature.

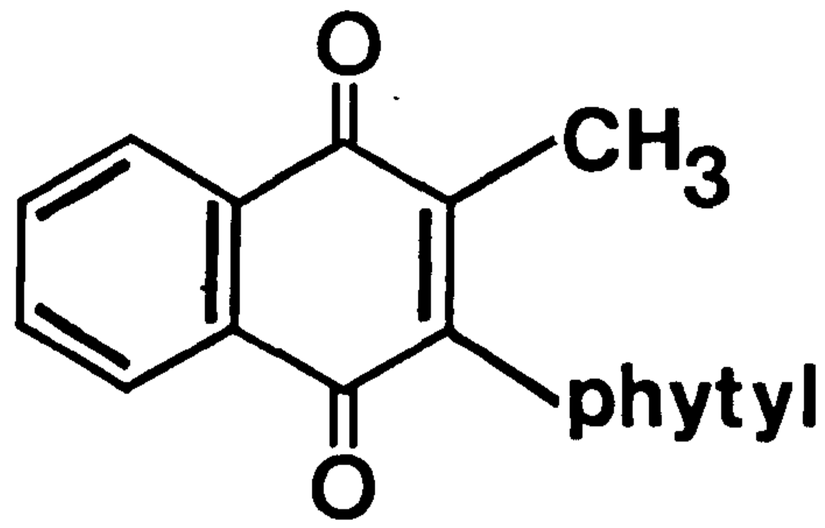
A. Vitamin K_1 (phylloquinone, 2-methyl-3-phytyl-1,4-naphthoquinone). This was initially isolated from the photosynthetic regions of higher plants and has a phytyl (20 carbon) side chain made up of four isoprene units linked together (Figure 1.2). Vitamin K_1 is administered as an antidote to patients who have become over anticoagulated on coumarin anticoagulant therapy, and to alleviate haemorrhagic disease in the newborn. In addition, this form of the vitamin is very effective in restoring clotting factor synthesis in chronically anticoagulated rabbits (Park and Leck, 1982).

B. Vitamin K_2

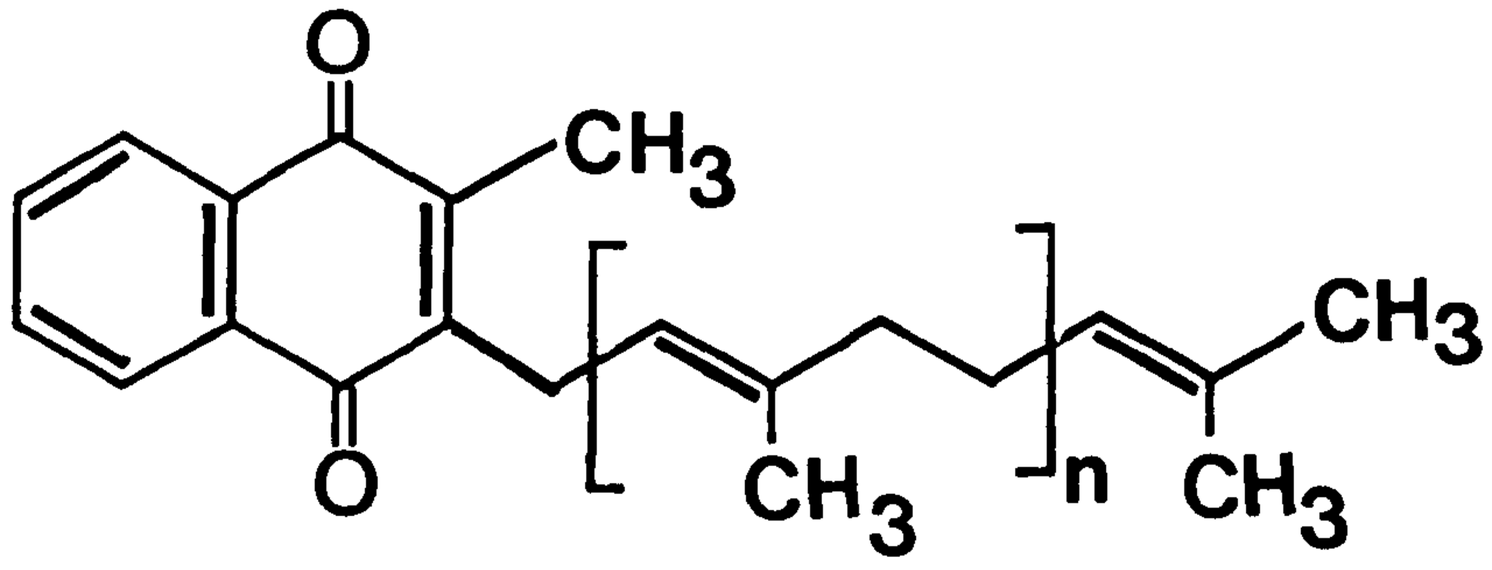
This consists of a series of compounds called the menaquinones which are produced solely by microorganisms (Pennock, 1966). Vitamin K_2 is produced by gut bacteria in man and animals. The side chain in the K_2 vitamins is of variable length, and gives rise to a number of compounds known as the MK-series (Figure 1.2). The number of isoprene units varies from 1 to 13, although only 4 to 13 are produced naturally The high molecular weight forms of vitamin K_2 are the storage forms of vitamin K_1 in man (Duello and Matschiner, 1972) and animals (Duello and Matschiner, 1971). MK-7 is especially prevalent as a storage form in man.

Figure 1.2 illustrates the structure of menadione. This synthetic form of the vitamin has no side chain and has relatively little biological activity. Nevertheless, the water soluble phosphate form

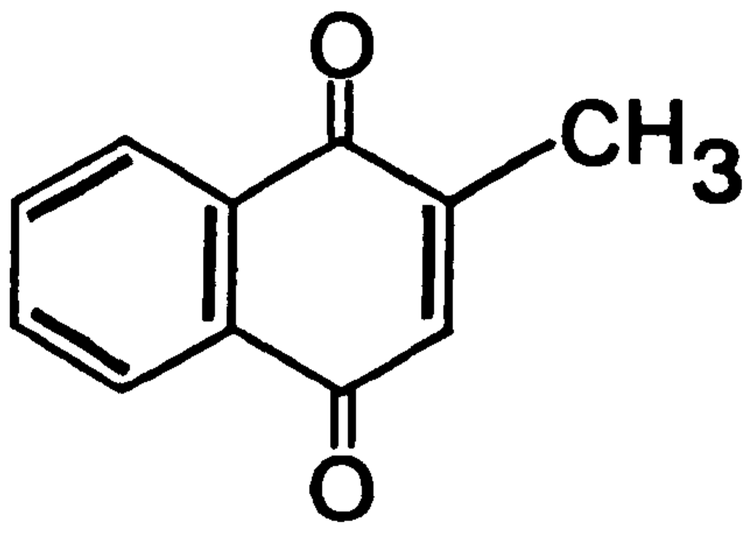
FIGURE 1.2 Chemical structures of vitamin K_1, vitamin K_2 and vitamin K_3.



Vitamin K_1



Vitamin K_2



Vitamin K_3 (menadione)

of this compound is administered for prophylaxis of neonatal haemorrhage. Menadione is inactive in the anticoagulated rabbit (Hart et al., 1984).

1.2.1 The Pharmacokinetics of Vitamin K

Vitamin K_1 and vitamin K_2 are absorbed by different mechanisms and in different areas of the gastrointestinal tract. Vitamin K_1 is absorbed from the small intestine by an energy dependent process which shows saturation kinetics (Hollander, 1973; Hollander et al., 1977). The menaquinones are synthesised by bacteria in the terminal ileum and large intestine, and are thought to be absorbed by passive diffusion in the large intestine (Hollander et al., 1976; Hollander et al., 1977).

It is uncertain whether vitamin K_1 from the diet, or vitamin K_2 from bacterial synthesis is the predominant form of vitamin K absorbed in vivo. Whatever the source, the minimum daily requirement of vitamin K is low and has been calculated to be 1 $\mu g\ kg^{-1}$ (Frick et al., 1967; Barkhan and Shearer, 1977).

As a consequence of the marked inter-individual variation in the availability of vitamin K observed in man following oral administration (Park et al., 1984), it is preferable to administer vitamin K intravenously in experimental situations.

Following the intravenous administration of vitamin K_1 to man, plasma concentrations of the vitamin decline bi-exponentially with an initial half-life of approximately 0.5 h, followed by a terminal half-life of 1 to 2 h (Shearer et al., 1972; Bjornsson et al., 1979; Park et al., 1984). A very similar plasma profile for vitamin K_1 has been demonstrated in the rabbit (Park et al., 1979; Park et al., 1980).

The major route of excretion of vitamin K_1 in man appears to be the biliary system, although urinary excretion contributes a significant amount to the excretion of the vitamin (Shearer et al., 1972). Two major metabolites of vitamin K_1, in which the phytyl side chain is shortened to either 5 or 7 carbon units, have been identified in urine after both oral and intravenous administration of the vitamin (Shearer and Barkhan, 1973). Both the urinary and the biliary metabolites are conjugated with glucuronic acid.

1.2.2 The Pharmacodynamics of Vitamin K

The Mechanisms of Blood Coagulation

Blood coagulation involves a series of enzymic conversions of serine protease zymogens to active serine proteases called clotting factors. Roman numerals are used for the nomenclature of these factors and the letter 'a' following a Roman numeral indicates an activated factor.

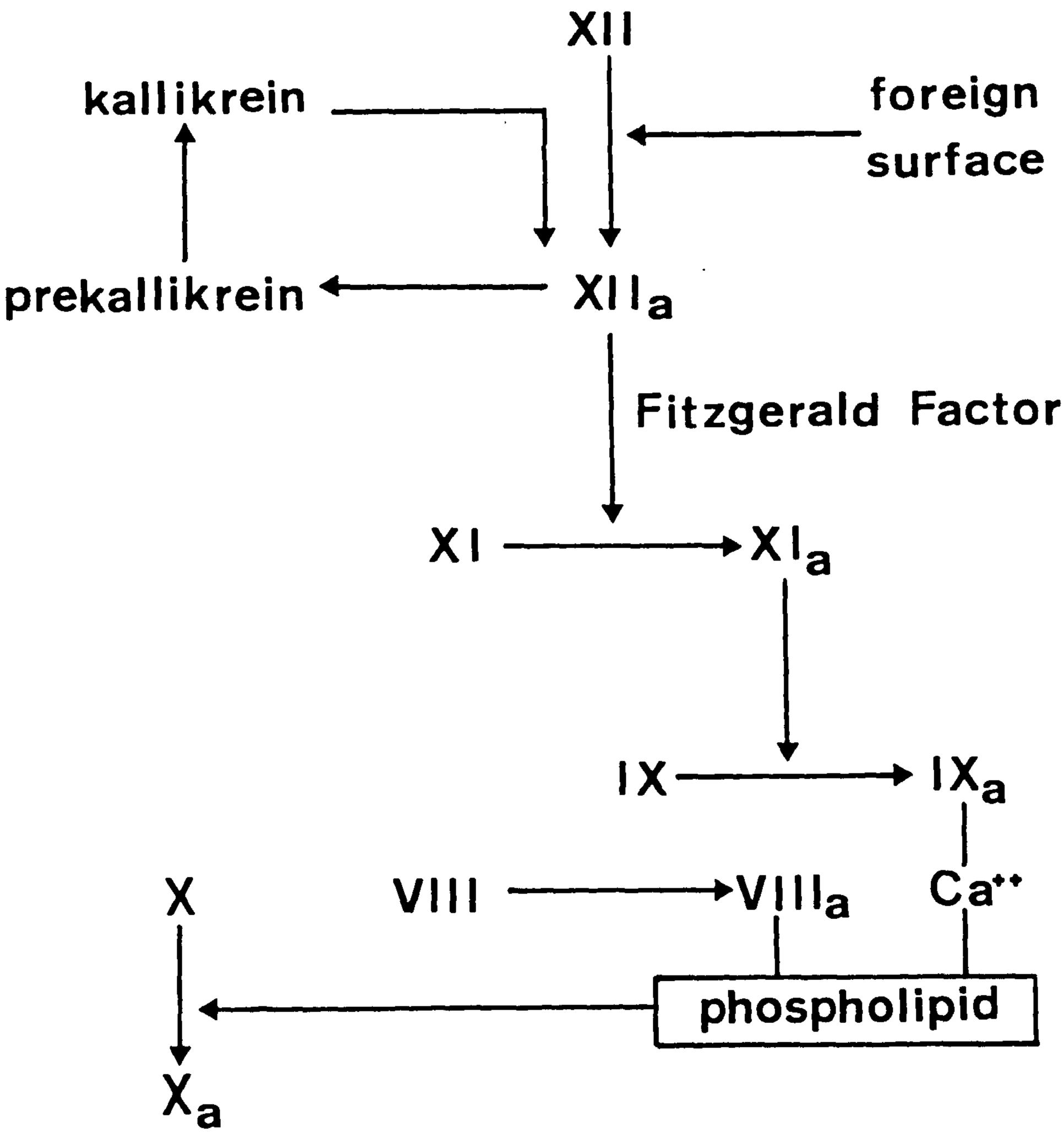
In the study of coagulation mechanisms, three pathways are distinguished, the intrinsic, the extrinsic and the common pathways. This distinction is of value to researchers but is artificial and there is no clear demarcation between the systems in physiological events.

The Intrinsic Pathway

Contact with a foreign surface is the initiating stimulus for the intrinsic pathway. A diagrammatic representation of this pathway is illustrated in Figure 1.3.

On contact with a surface, factor XII undergoes a confirmational change to acquire enzymic activity. Factor XIIa subsequently converts

FIGURE 1.3 The Intrinsic Pathway of Blood Coagulation



some prekallikrein to kallikrein, which in turn rapidly activates more factor XII by a positive feedback mechanism (Cochrane et al., 1973) and thus amplifies the effect of surface contact. The rapid activation of factor XII involves the proteolytic cleavage of factor XII to yield fragment E (Revak and Cochrane, 1976). Fragment E in the presence of an essential co-factor called Fitzgerald Factor (Kininogen II), leads to the calcium independent activation of factor XI (Saito et al., 1975). Factor IXa is responsible for the conversion of factor IX to factor IXa. This is a calcium dependent process (Kato et al., 1974). Factor IXa then activates factor X, in a reaction which requires phospholipid, calcium and factor VIII. Factor VIII has no enzymic activity, but functions as a catalyst enhancing the rate of factor X activation.

The Extrinsic Pathway

This pathway is initiated by blood coming into contact with thromboplastin which is released when tissue is damaged. A diagrammatic representation of this pathway is illustrated in Figure 1.4.

In the presence of the lipoprotein complex, thromboplastin and calcium, factor VII is activated. A complex of factor VIIa with thromboplastin is required for the activation of X.

The Common Pathway

The intrinsic and the extrinsic pathways of blood coagulation converge with the activation of factor X. The common pathway is illustrated in Figure 1.5, and is the sequence of events following factor X activation which lead to the formation of the fibrin clot.

A complex, which requires calcium and phospholipid for its activation, is formed between activated factor X and factor V. In

FIGURE 1.4 The Extrinsic Pathway of Blood Coagulation.

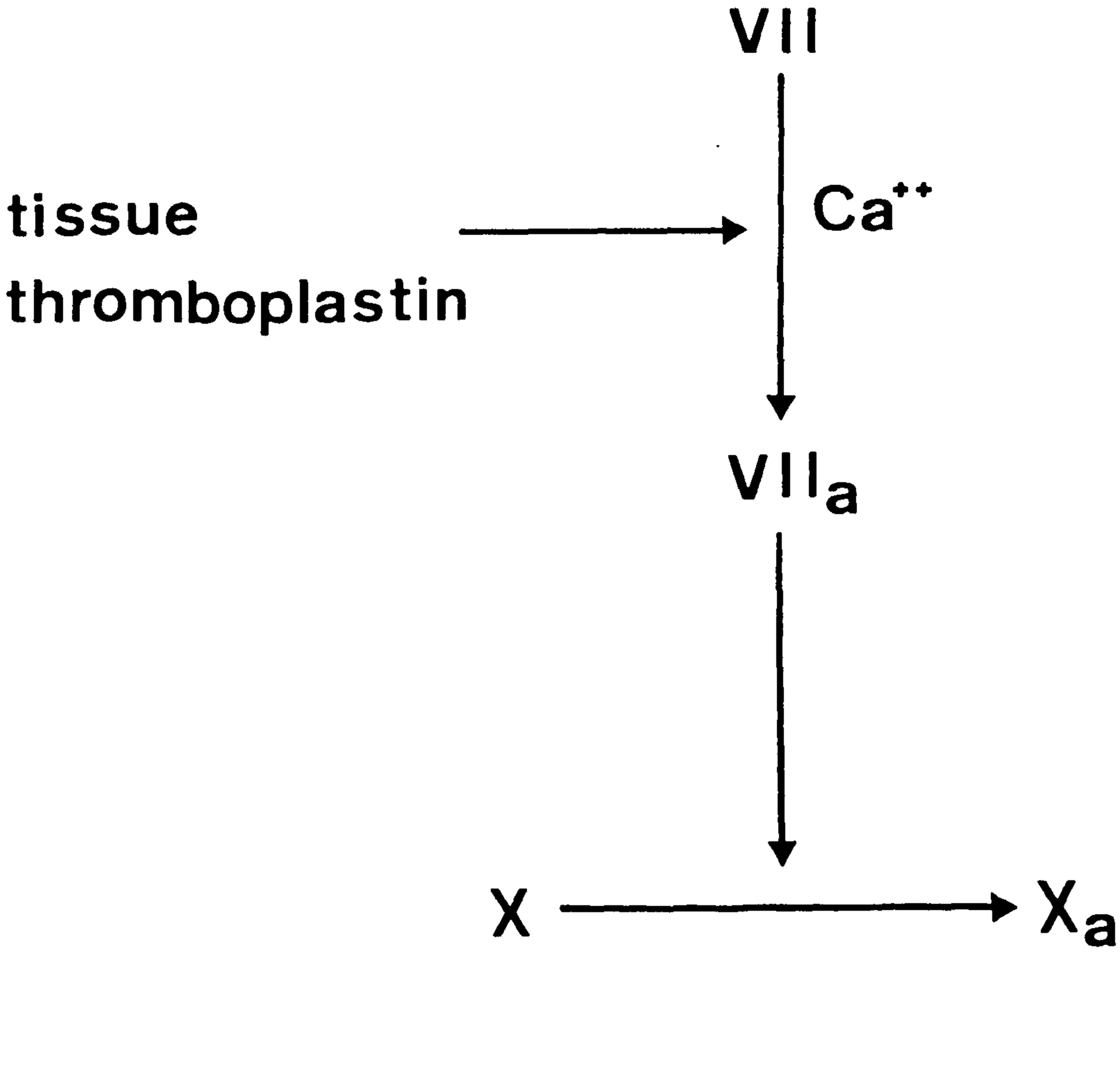
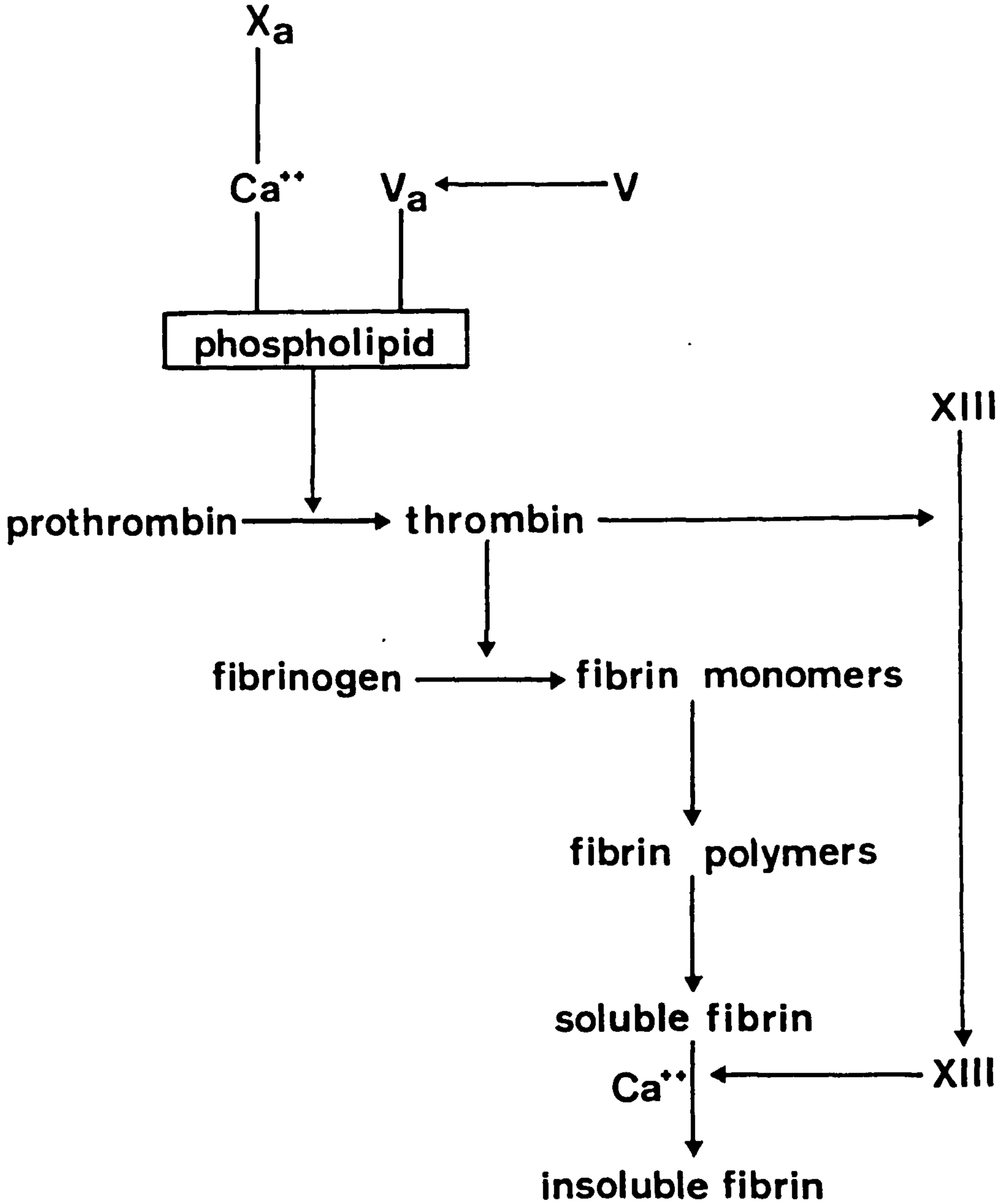


FIGURE 1.5 The Common Pathway of Blood Coagulation.



the presence of this complex, prothrombin (factor II) is cleaved to yield thrombin and an inert fragment. Thrombin is a serine protease which interacts with fibrinogen to form fibrin monomers. The fibrin monomers polymerise end to end to form fibrils and side to side to make coarser fibres. The initial fibrin polymer constitutes a soft clot which is readily dissolved if the process proceeds no further. However, factor XIII, the fibrin stabilizing factor catalyses the formation of stronger cross-links between the monomer units. Thus the fibrin clot becomes insoluble and resistant to proteolytic degradation.

The blood coagulation system provides a biochemical amplification of the initial stimulus through a cascade of six sequential, enzymatically catalysed steps. If it is assumed that there is a ten fold amplification factor at each stage, the amplification factor for the whole process is one million.

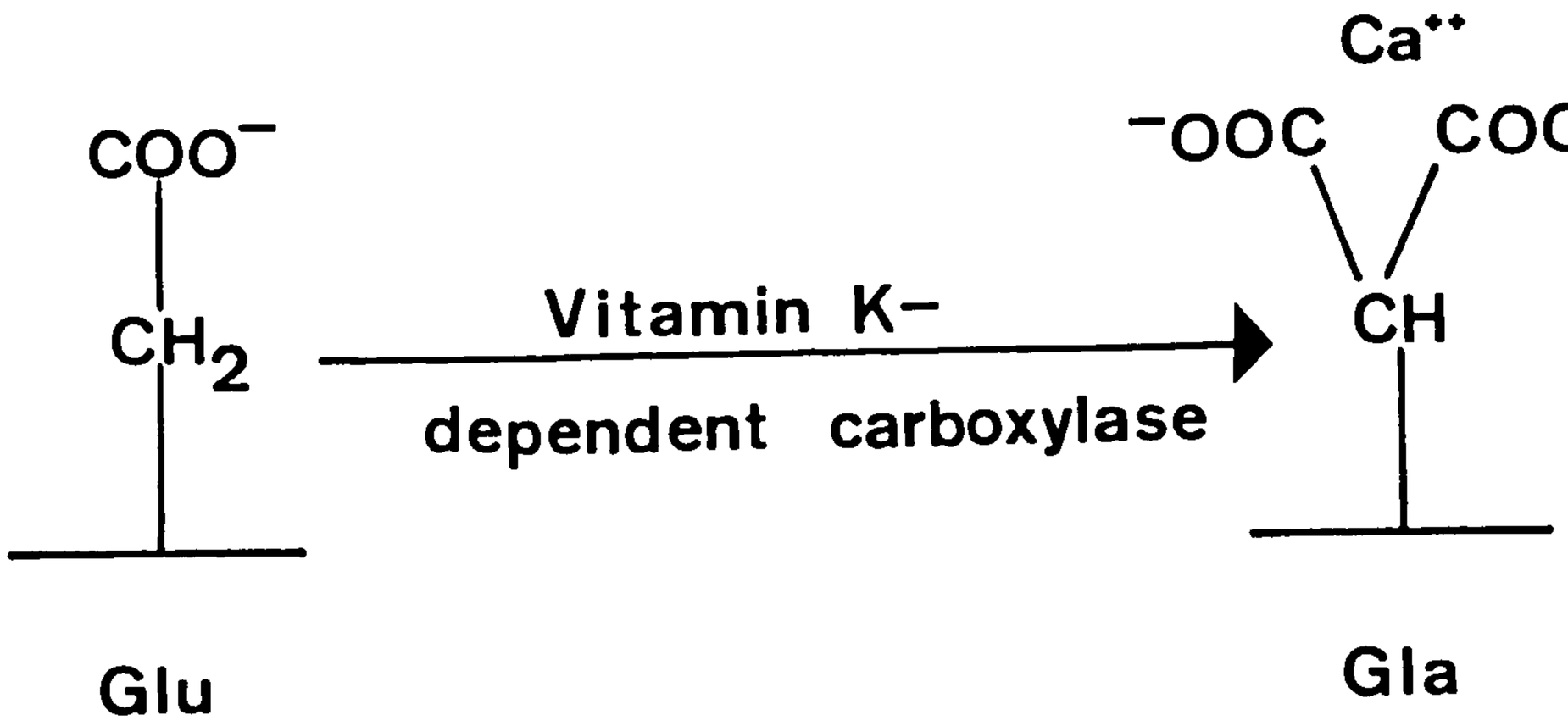
The Role of Vitamin K in Blood Coagulation

Shortly after its discovery and characterization, the deficiency of vitamin K was shown to be associated with a marked decrease in the activity of clotting factors II, VII, IX and X (Warner et al., 1938); Owen, 1947) but the role of the vitamin was not defined.

A breakthrough came in 1963, when Hemker et al., postulated that blood from patients receiving oral anticoagulant therapy contained a mixture of normal and abnormal prothrombin. The abnormal prothrombin was termed Protein Induced by Vitamin K Antagonists (or Absence) or PIVKA, and at that time it was regarded as a precursor of prothrombin. In 1968, Ganrot and Nilehn demonstrated the presence of abnormal prothrombin in the plasma of patients taking coumarin anticoagulants.

The abnormal prothrombin was subsequently identified immunochemically in the plasma of dicoumarol treated patients (Josso et al., 1968; Ganrot and Nilehn, 1968) and cattle (Reekers et al., 1973). PIVKAs have also been identified in the livers of rats and rabbits (Suttie, 1972). The abnormal prothrombin was shown to have the same molecular weight, carbohydrate and amino acid composition as prothrombin and cross-reacted with the antibody to prothrombin (Stenflo, 1970). In the presence of calcium, the electrophoretic migration rate of abnormal prothrombin was different to that of normal prothrombin. It was subsequently shown that whereas normal prothrombin has the ability to bind calcium strongly, the abnormal prothrombin lacks this ability (Nelsestuen and Suttie, 1972; Stenflo and Ganrot, 1973). In 1974, two groups (Stenflo et al; Nelsestuen et al.) independently reported that the amino terminal portion of prothrombin contained a novel amino acid, γ - carboxyglutamic acid (Gla), and that these residues were reduced or absent in abnormal prothrombin and replaced by glutamic acid (Glu) residues. Furthermore it was demonstrated that factors VII, IX and X, the other vitamin K - dependent proteins also contain Gla (Howard and Nelsestuen, 1974; Bucher et al., 1976). Thus it was proposed that Glu residues are present in the precursors of clotting factors II, VII, IX and X, and that a vitamin K - dependent reaction converts them to Gla residues and therefore imparts a strong calcium binding activity to these factors (Figure 1.6). Several workers (Hill et al., 1968; Bell and Matschiner, 1969; Suttie, 1970) provided good evidence that the vitamin K - dependent modification was a post-translational reaction. When rats deficient in prothrombin activity, due to either a lack of dietary vitamin K or coumarin anticoagulation, were given vitamin K_1, an early release of prothrombin was seen. This response was only slightly inhibited by prior administration of the

FIGURE 1.6 Vitamin K - dependent carboxylation of a glutamic acid residue (Glu) to a γ-carboxyglutamic acid residue (Gla)



protein synthesis inhibitor, cycloheximide. Furthermore, Suttie and Shah (1971) found that administration of a mixture of radiolabelled amino acids and vitamin K_1 to vitamin K deficient rats resulted in prothrombin which contained no radiolabel. These observations suggested that protein synthesis was not required for the vitamin K-dependent step in the synthesis of prothrombin and that the prothrombin did not contain newly synthesized protein. Furthermore, it appeared that vitamin K was involved in the modification of hepatic prothrombin precursors from a pre-existing pool. These observations led to the discovery of an enzymatic activity in rat liver microsomal preparations dependent on vitamin K which would promote the incorporation of $H^{14}CO_3^-$ into the precursor proteins (Esmon et al., 1975). We now know that the enzyme vitamin K-dependent carboxylase is responsible for the conversion of Glu residues to Gla residues (Sadowski et al., 1976).

The conversion of Glu residues to Gla residues provides the clotting factors with a strong calcium binding property which enables the protein to interact with negatively charged phospholipid surfaces. This provides a high concentration of enzyme and substrate in the two phospholipid-dependent reactions of the clotting system (Figure 1.3 and Figure 1.4).

It is now well established that PIVKAs are non- or partially-carboxylated clotting factor precursors (Magnusson et al., 1974; Stenflo et al., 1974). There is evidence that non-functional forms of the other vitamin K-dependent clotting factors are also present in vitamin K deficiency and hypoprothrombinaemia (Jackson and Suttie, 1977). It is thought that at least 7 out of a possible 10 Glu residues must be carboxylated for biological activity in clotting factors II and VII (Esnouf and Prowse, 1977).

In addition to the four vitamin K-dependent clotting factors (Figure 1.7) normal plasma contains several other vitamin K-dependent proteins which possess Gla residues. These include proteins C and S, which are involved in blood coagulation, but unlike factors II, VII, IX and X, these proteins are involved in anticoagulant mechanisms.

The Vitamin K-Epoxide Cycle

The activation of the vitamin K-dependent proteins involved in blood clotting mechanisms requires the vitamin K-dependent carboxylase enzyme. During this process, vitamin K_1 is converted to the biologically inactive metabolite, vitamin K_1 2,3-epoxide (Willingham and Matschiner, 1974) and thus the production of Gla residues in clotting factors appears to be coupled to the epoxidation of the vitamin (Larson *et al.*, 1981). Vitamin K_1 2,3-epoxide is reduced back to vitamin K_1 quinone by the enzyme vitamin K-epoxide reductase (Matschiner *et al.*, 1974). The quinone form of vitamin K_1 must be reduced to the hydroquinone before it can act as a co-factor in the carboxylation reaction. Vitamin K quinone reductase is the enzyme responsible for the production of the hydroquinone.

The cyclic conversion of vitamin K_1 and vitamin K_1 2,3-epoxide is known as the vitamin K-epoxide cycle (Figure 1.8) and is essential for normal clotting factor synthesis (Bell and Matschiner, 1972).

Vitamin K-Dependent Carboxylase

Vitamin K-dependent carboxylase catalyses the post-translational carboxylation of glutamic acid (Glu) residues to yield γ-carboxyglutamic acid (Gla) residues in a variety of proteins. It is thought to be a lumenal membrane protein which is predominantly located in the

FIGURE 1.7 Outline of the blood coagulation mechanisms. The vitamin K-dependent clotting factors are underlined.

pl - phospholipid

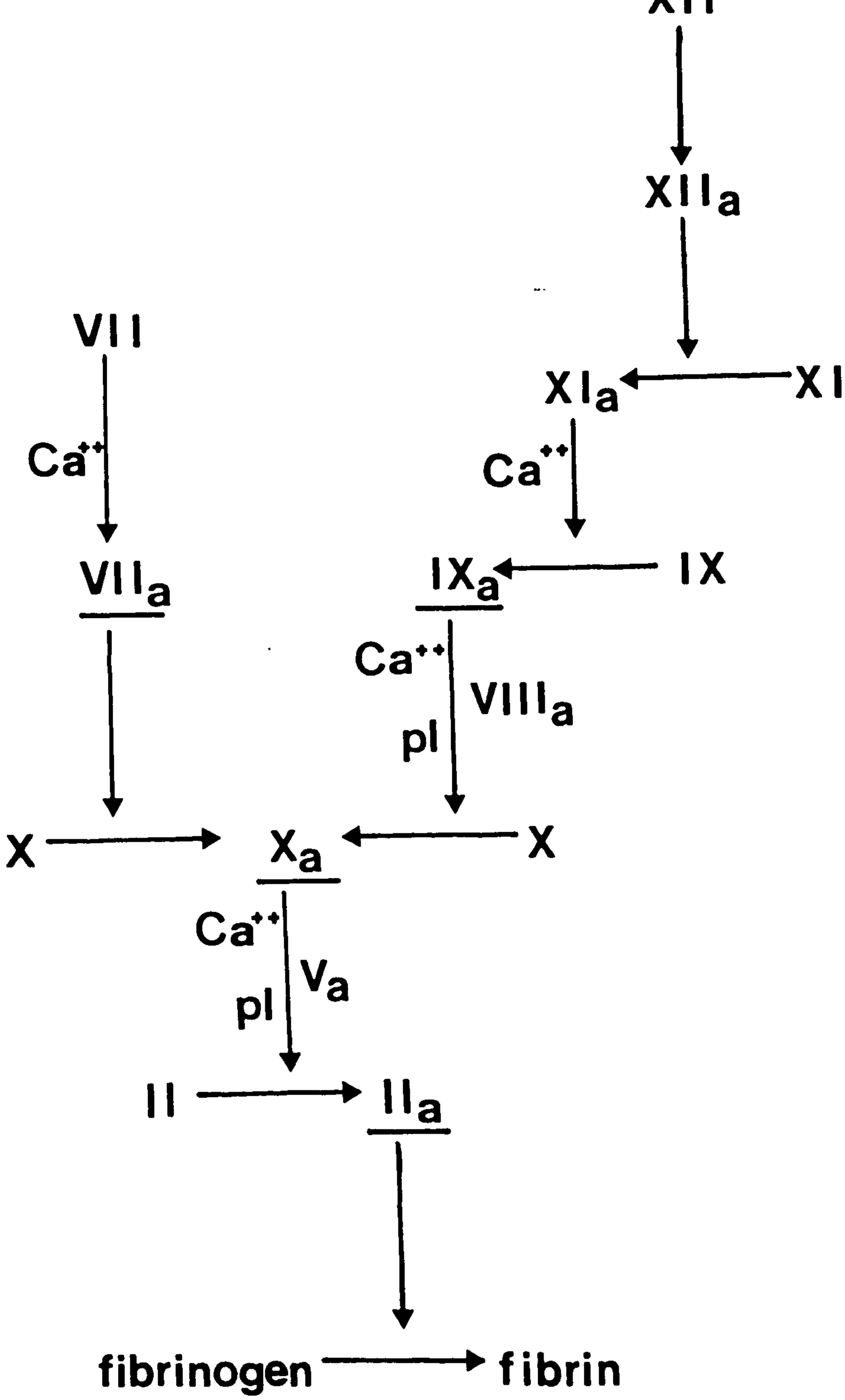
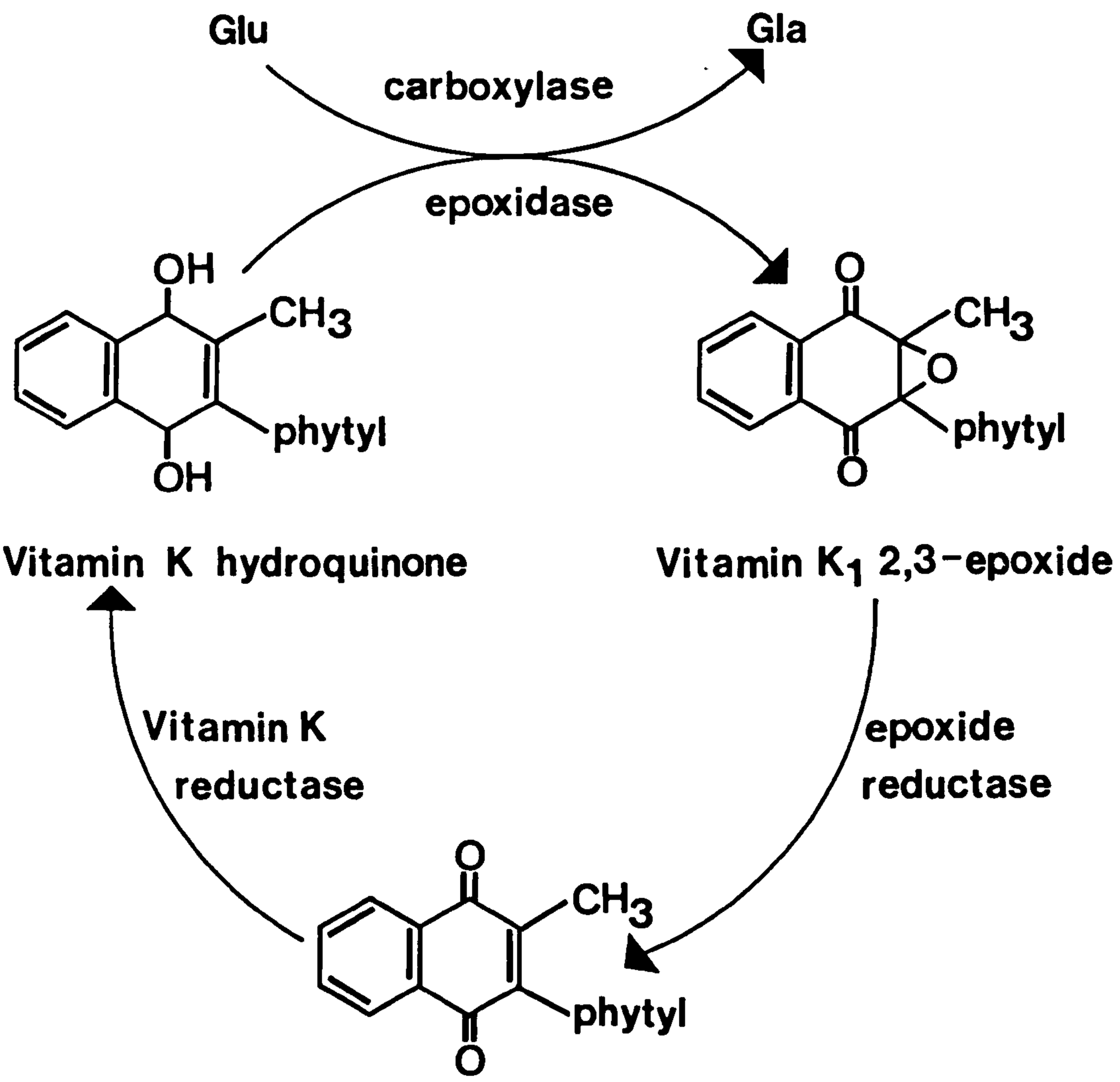


FIGURE 1.8 The vitamin K-epoxide cycle.

Glu - glutamic acid

Gla - γ - carboxyglutamic acid



rough endoplasic reticulum of hepatocytes but is also present in the smooth endoplasmic reticulum (Carlise and Suttie, 1980). The enzyme requires vitamin K_1 hydroquinone or vitamin K_1 quinone in the presence of reducing agent such as NAD(P)H or dithiothreitol, molecular oxygen and carbon dioxide for its activity.

Consistent with the vitamin K - epoxide cycle, the liver contains both vitamin K_1 and vitamin K_1 2,3-epoxide (Matschiner et al., 1970). The enzyme responsible for the generation of the epoxide is called vitamin K epoxidase. Vitamin K_1 2,3-epoxide is produced concomitantly with the production of carboxylated products of the vitamin K - dependent carboxylase mediated reaction (Suttie, 1980). Thus it has been suggested that the epoxidase and the carboxylase reactions are in some way coupled. Much difficulty has been experienced in the purification and isolation of the carboxylase and epoxidase activity and this has prevented the clear determination of the relationship between these two enzymes.

There is considerable evidence to support the theory that carboxylation and epoxidation are linked. Many requirements for the optimal function of these two enzymes are similar (Sadowski et al., 1979), the subcellular location of both enzymes is the same (Willingham and Matschiner, 1974), and it has been proposed that both reactions proceed via vitamin K_1 hydroperoxide (Larson and Suttie, 1978; Demetz et al., 1982). Furthermore, the formation of vitamin K_1 2,3-epoxide is elevated in rat livers containing increased concentrations of endogenous substrates for carboxylation (Zimmerman and Matschiner, 1974). However, in most cases on a molar basis, more epoxide is formed than carbon dioxide incorporated into enzyme substrate (Suttie et al., 1980). Thus, epoxidation can take place without carboxylation. In contrast, carboxylation without epoxidation has never been demonstrated.

Vitamin K Epoxide Reductase and Vitamin K Reductase

For continuous clotting factor synthesis, the vitamin K_1 2,3-epoxide produced during the γ-carboxylation of Glu residues, has to be converted back to vitamin K_1 quinone. This reaction is catalysed by the enzyme, vitamin K epoxide reductase (Zimmerman and Matschiner, 1974) which is present in liver microsomal preparations. The addition of a cytosolic fraction appears to be important for the activity of the enzyme (Siegfried, 1979). It is thought that this provides a reducing agent whose activity can be mimicked, to a certain extent *in vitro*, by the addition of dithiothreitol (DTT). (Whitlon *et al.*, 1978; Hildebrandt and Suttie, 1982). The nature of the *in vivo* reducing agent and the pathway by which it is produced have not yet been determined.

Vitamin K_1 hydroquinone which is essential for the carboxylation of Glu-residues, is produced by the reduction of vitamin K_1 quinone. This reaction has been shown to be catalysed by DT diaphorase (NAD(P)H dehydrogenase, EC1,6.99.2), an enzyme which occurs primarily in the cytoplasm of hepatocytes (Wallin *et al.*, 1978; Wallin and Suttie, 1981) and/or by a microsomal reductase enzyme (Whitlon *et al.*, 1978; Fasco and Principe, 1980).

The mechanisms of vitamin K_1 quinone and vitamin K_1 2,3-epoxide reduction have not yet been elucidated, primarily because the enzymes involved in these reactions have not yet been purified. At present it is not known whether these reactions are catalysed at the same site on a single enzyme, at separate sites on a single enzyme, or at separate sites on two distinct enzymes.

Current evidence suggests that the reduction of the epoxide and the quinone are catalysed by the same enzyme. Both enzymes have been

shown to require sulphydryl compounds as co-factors for their activity (Zimmerman and Matschiner, 1974; Fasco and Principe, 1980) and both can be inhibited by 4-hydroxycoumarin anticoagulants such as warfarin (Whitlon et al., 1978; Hildebrandt and Suttie, 1982). The action of warfarin on the vitamin K-epoxide cycle will be discussed later in this chapter. In addition, vitamin K_1 quinone is an effective inhibitor of vitamin K_1 2,3-epoxide reduction in rat liver microsomes (Preusch and Suttie, 1984). This further substantiates the theory that the two reductase enzymes, are in fact either a single enzyme or a multisite enzyme complex.

1.3 The Pharmacology of Warfarin

One of the major problems associated with the use of warfarin in anticoagulant therapy, is the inter-individual variation in pharmacological response to this compound (Breckenridge, 1977). This can be attributed to variation in both the pharmacokinetics and the pharmacodynamics of warfarin.

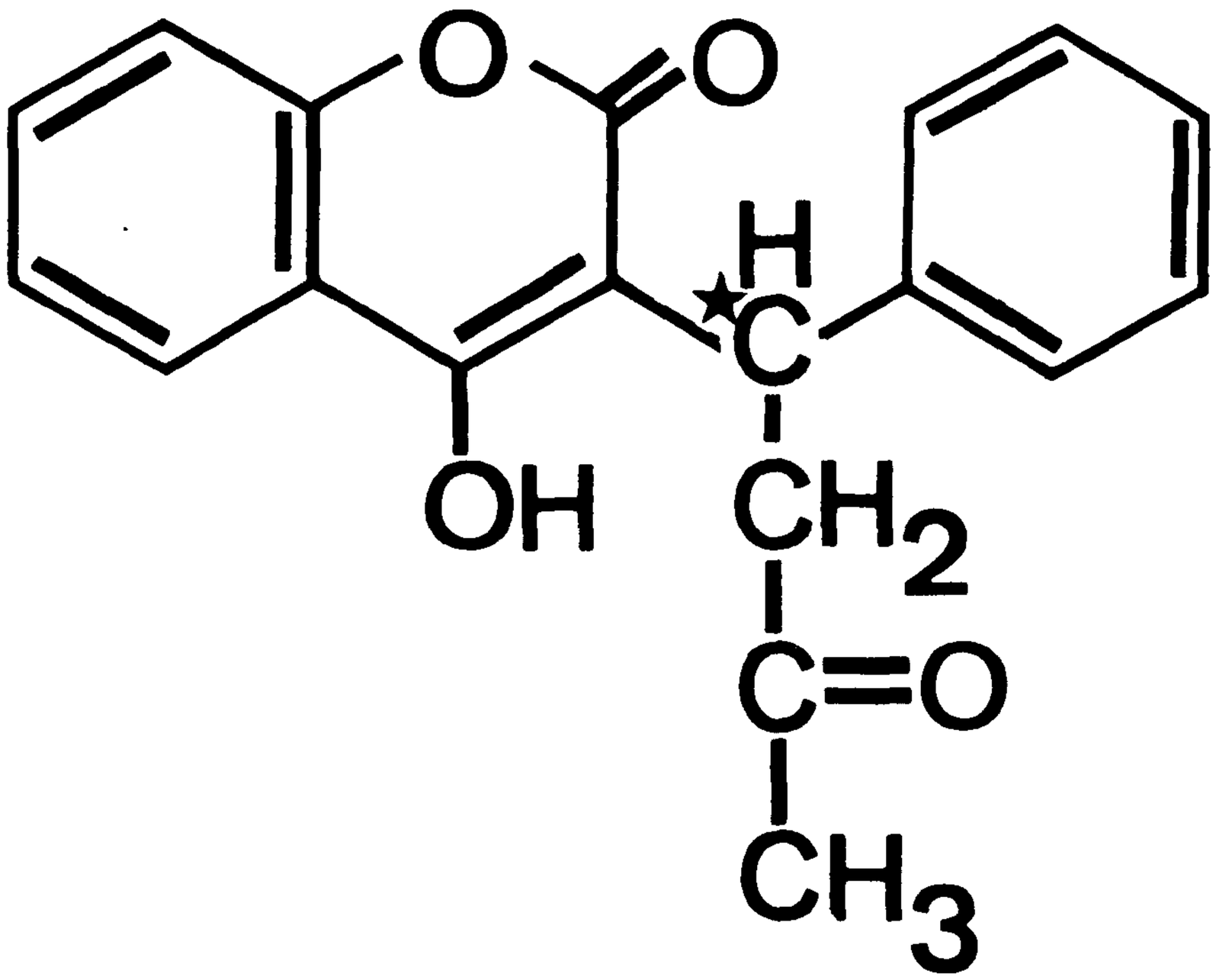
1.3.1 The Pharmacokinetics of Warfarin

Warfarin is the most studied of the 4-hydroxycoumarin anticoagulants in both man and experimental animals, particularly the rat. It possesses an asymmetric carbon atom, and thus exists as two structurally distinct forms, the enantiomers R(+) warfarin and S(-) warfarin (Figure 1.9). When administered clinically, warfarin is given as equal amounts of the enantiomers - a racemic mixture.

Racemic warfarin is rapidly and almost completely absorbed from the stomach and the upper gastrointestinal tract (O'Reilly *et al*, 1963; Breckenridge and Orme, 1973). The time to peak plasma concentrations is relatively long (3 to 9h; O'Reilly *et al*., 1963). There is no evidence for differential absorption rates between the enantiomers of warfarin.

In both man and the rat, the plasma protein binding of racemic warfarin has been shown to be approximately 99% (Yacobi and Levy, 1977). Virtually all the binding appears to be to plasma albumin, and in man the degree of binding is constant over the normal therapeutic range of plasma concentrations. Contrasting results have been obtained concerning the degree of binding of the enantiomers of warfarin in man. Equilibrium dialysis at 27^{o}C, has demonstrated the same degree of protein

FIGURE 1.9 Chemical structure of warfarin ★ indicates the asymmetric carbon atom.



binding for both R(+) and S(-) warfarin (O'Reilly, 1971). However, using the same method at 37°C, S(-) warfarin has been shown to be more highly protein bound than R(+) warfarin (Sellers and Koch-Weser, 1975). S(-) warfarin appears to be more highly protein bound than R(+) warfarin in rat plasma (Yacobi and Levy, 1977).

Warfarin distributes into a relatively small apparent volume of distribution of about 10 l 70 kg^{-1} in man, because of extensive protein binding (Breckenridge and Orme, 1973; Lewis et al., 1974). Using a one compartment model and assuming complete bioavailability, estimates of the apparent volume of distribution for the enantiomers of warfarin and the racemate are similar (Breckenridge et al., 1974; Hignite et al., 1980; Banfield et al., 1983). The apparent volume of distribution of warfarin in the rat appears to be similar to that in man on a weight related basis (Breckenridge and Orme, 1972, Yacobi et al., 1974; Yacobi et al., 1984).

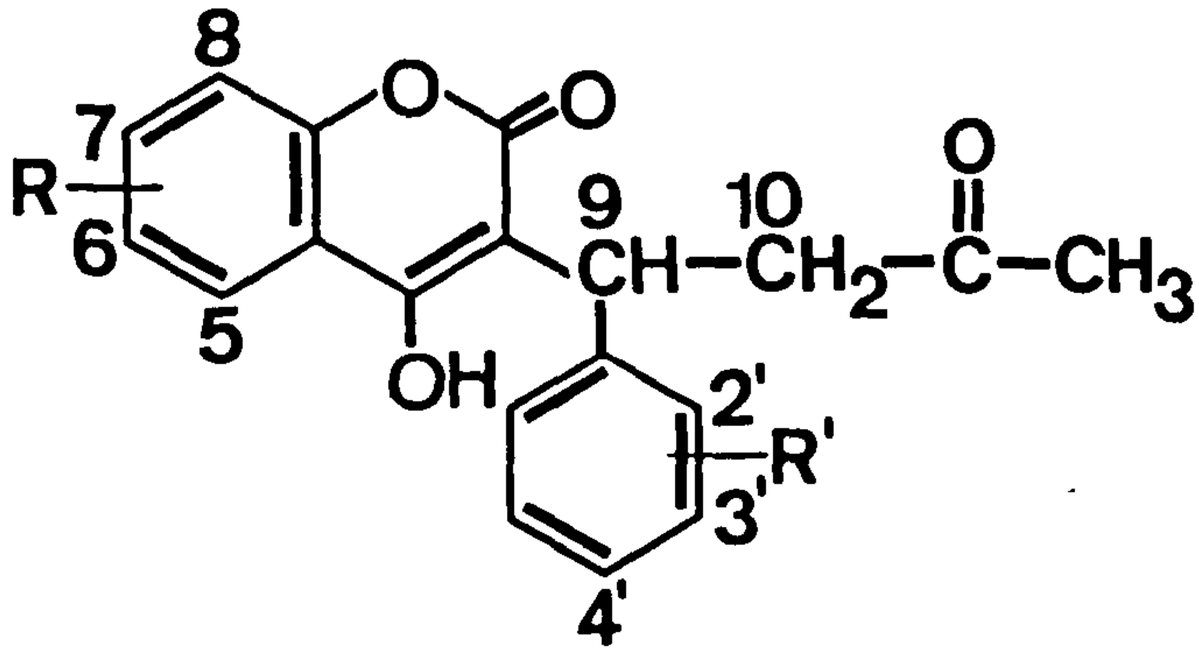
Warfarin is eliminated almost entirely by metabolism, only traces of the parent compound are excreted in urine (Banfield et al., 1983). Plasma clearance is very low, typically 0.2 l h^{-1} 70 kg^{-1} (Breckenridge et al., 1974; Wingard et al., 1978), and hepatic extraction is low. The effect of hepatic blood flow per se on warfarin clearance is thought to be negligible since warfarin undergoes capacity limited, binding sensitive elimination. The clearance of S(-) warfarin is significantly greater than that of R(+) warfarin, both when administered as the individual enantiomers (Breckenridge et al., 1974; Wingard et al., 1978) or when administered as the racemate (Banfield et al., 1983). These findings indicate that there is no pharmacokinetic interaction between R(+) and S(-) warfarin (Levy et al., 1978).

In direct contrast to this observation in man, the plasma clearance of R(+) warfarin is significantly greater than that of S(-) warfarin

in the rat (Yacobi et al., 1984). The plasma clearance of racemic warfarin in rats is considerably lower than that in man (Yacobi et al., 1974). The plasma half-life of warfarin has been determined in man, in many studies, and considerable inter-individual variation has been observed. Most studies report mean half-lives of between 35 and 45 h, however half-lives as low as 15 h and as high as 80 h have been reported (O'Reilly et al., 1963; O'Reilly and Aggeler 1968). The half-life of warfarin in man appears to be independent of dose over a dose range of 50-2000 mg (O'Reilly et al., 1971; Hackett et al., 1985). The half-life of R(+) warfarin is significantly longer than that of S(-) warfarin (Breckenridge et al., 1974; O'Reilly, 1974) as would have been predicted from the apparent volume of distribution and clearance data previously discussed. In contrast in the rat, the half-life of S(-) warfarin is significantly longer than that of R(+) warfarin (Breckenridge and Orme, 1972; Yacobi et al., 1984).

The differences in the pharmacokinetics of the enantiomers of warfarin appear to be due to differences in their routes of metabolism. Warfarin is primarily metabolised by the hepatic microsomal mixed function oxidase system, although the reduction products of warfarin metabolism are formed by cytosolic enzymes (Hewick and Moreland, 1975). Figure 1.10 illustrates the identified metabolites of warfarin in man. In vivo studies have demonstrated that R(+) warfarin is primarily oxidised to 6-hydroxywarfarin, but it also reduced at its carbonyl group to an optically active alcohol, R, S warfarin alcohol and oxidised to 7-hydroxywarfarin. In contrast, the major metabolite of S(-) warfarin is 7-hydroxywarfarin with small amounts of the optically active alcohol, S,S warfarin alcohol and 6-hydroxywarfarin also being produced (Hewick and McEwen, 1973; Lewis et al., 1974; Banfield et al., 1983).

FIGURE 1.10 Metabolites of warfarin in man.



R=R'=H

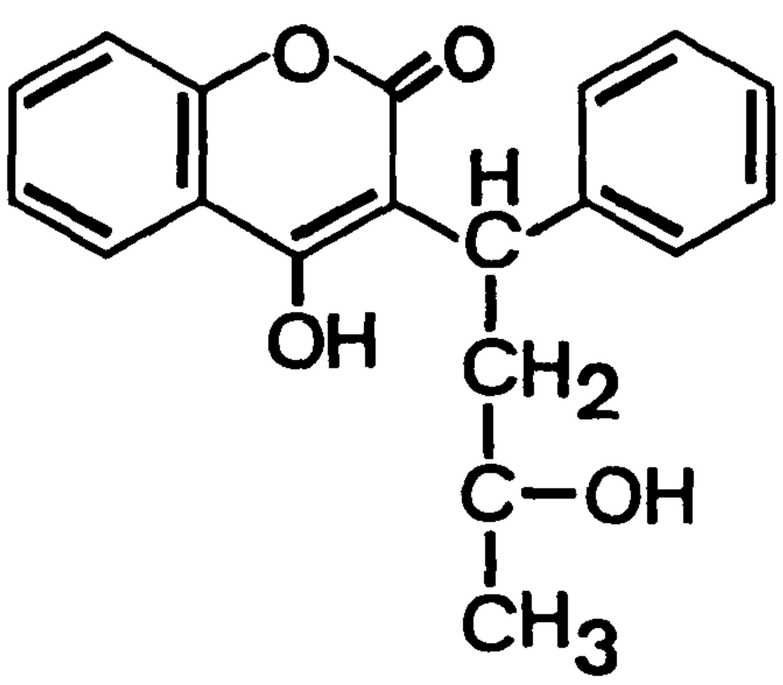
R=6-OH, R'=H

R=7-OH, R'=H

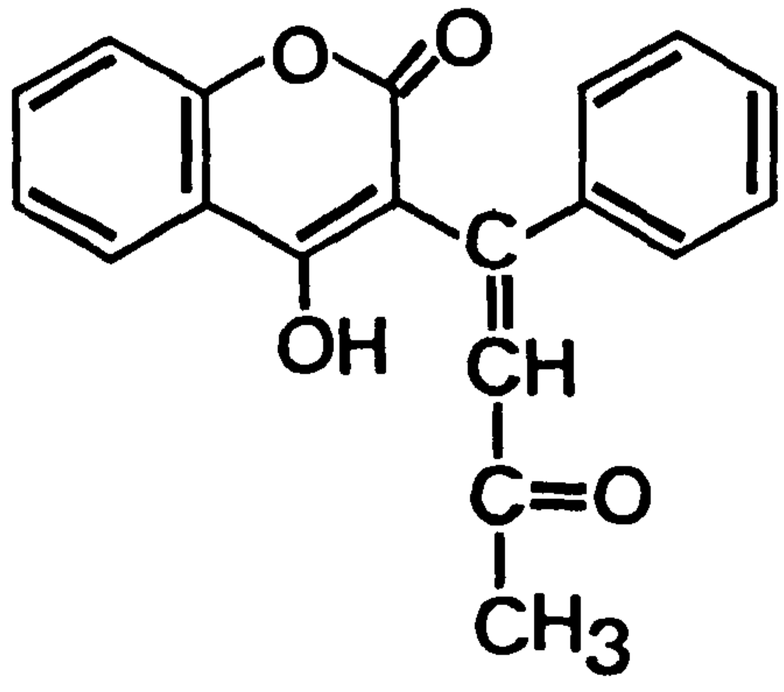
R=8-OH, R'=H

R=10-OH, R'=H

R=H, R'=4'-OH



Warfarin alcohols-
R,S ; S,S



Dehydrowarfarin

In vitro work using human liver microsomes, illustrated that both R(+) and S(-) warfarin are metabolised to dehydrowarfarin, 4', 6, 7, 8 and 10 hydroxywarfarin. The rate of formation of 6-hydroxy-warfarin was greater than that for the other metabolites from R(+) warfarin, whereas 7-hydroxywarfarin was formed more rapidly from S(-) warfarin (Kaminsky et al., 1984).

4-hydroxycoumarin anticoagulants other than warfarin are used both therapeutically and as rodenticides. Table 1.1 compares the plasma half-lives of these compounds with that of warfarin.

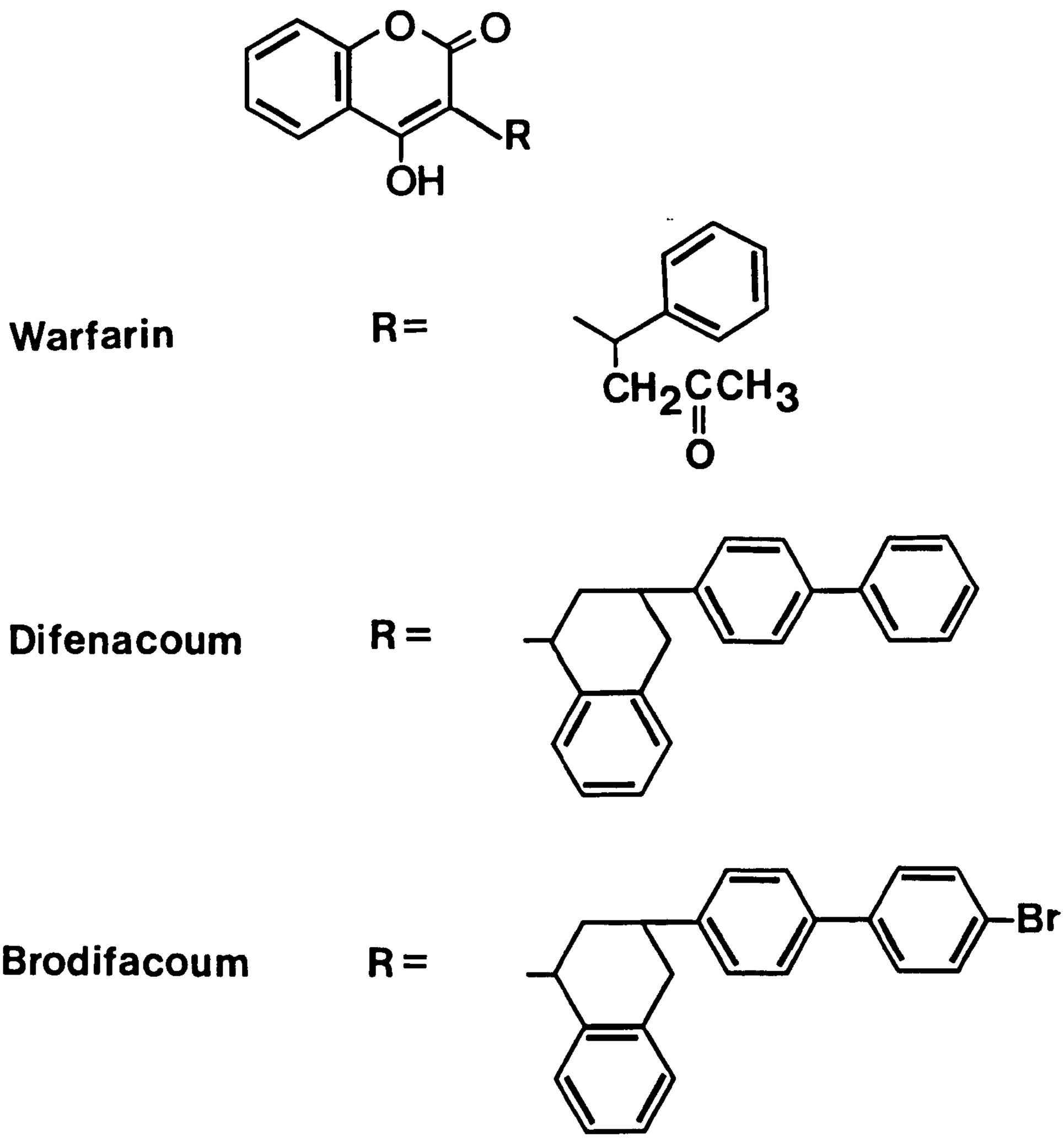
Phenprocoumon, which is the oral anticoagulant of choice in continental Europe, has a plasma half-life of approximately 160 h and the effect of a single dose lasts for 6 or more days (Jähnchen et al., 1976). In contrast, acenocoumarol is a short acting anticoagulant (O'Reilly, 1980). It has a plasma half-life of between 8 to 12 h (Dieterle et al., 1977; Thijssen and Baars, 1983) and as a consequence recovery from the anticoagulant effect is rapid.

Brodifacoum and difenacoum were developed as rodenticides for use against warfarin resistant rats (Hadler and Shadbolt, 1975). They possess the same 4-hydroxycoumarin nucleus as warfarin, however they differ in that they possess a novel tetrahydronapthyl side chain (Figure 1.11). Brodifacoum and difenacoum are more potent than warfarin in man (Barlow et al., 1982), the rat (Hadler and Shadbolt, 1975), the mouse (Balasubramanyam et al., 1984) and the rabbit (Park and Leck, 1982). In man, the plasma half-lives of these compounds are considerably longer than those of the 4-hydroxycoumarins administered for the treatment of thrombo-embolic disorders. As a consequence of this, the pharmacological effects of brodifacoum and difenacoum are very persistent when taken either purposefully in a suicide attempt (Barlow et al.,

TABLE 1.1 The plasma half-lives of 4-hydroxycoumarin anticoagulants in man.

Drug	Plasma half-life (h)
Warfarin	35 - 45
Acenocoumarol	8 - 12
Phenprocoumon	∿ 160
Brodifacoum	487

FIGURE 1.11 Chemical structures of warfarin, difenacoum and brodifacoum.



1982) or accidentally (Park et al., 1986). The greater potency of brodifacoum and difenacoum when compared to warfarin, make these novel 4-hydroxycoumarin anticoagulants useful pharmacological tools for studies in experimental animals.

It has been proposed that both therapeutic and rodenticidal 4-hydroxycoumarin anticoagulants exert their pharmacological effect by inhibition of the enzyme vitamin K epoxide reductase. Thus it is thought that differences in the pharmacokinetics but not in the pharmacodynamics, are largely responsible for the differences in the pharmacological effect of these compounds.

1.3.2 The Pharmacodynamics of Warfarin

In 1970 Matschiner and co-workers demonstrated the metabolism of vitamin K_1 to vitamin K_1 2,3-epoxide. In the normal situation, approximately 10% of vitamin K_1 in rat liver is present as the epoxide. However, during anticoagulant therapy it is possible for vitamin K_1 2, 3-epoxide to become the predominant form of the vitamin (Bell and Matschiner, 1970). Furthermore, in the presence of 4-hydroxycoumarin anticoagulants, a similar increase in the ratio of vitamin K_1 2,3-epoxide to vitamin K_1 was observed in the plasma of the rat and the rabbit (Bell and Matschiner, 1972; Shearer et al., 1973; Willingham et al., 1976; Park et al., 1979). Perhaps most importantly, with respect to anticoagulant therapy, vitamin K_1 2,3-epoxide was detected in the plasma of healthy volunteers anticoagulated with warfarin, following a physiological dose of the vitamin (Shearer et al., 1977). In vitro studies have illustrated that vitamin K_1 2,3-epoxide can be reduced to vitamin K_1 by the enzyme vitamin K epoxide reductase and that this enzyme is very susceptible to inhibition by coumarin anti-

coagulants such as warfarin (Bell et al., 1972; Whitlon et al., 1978; Hildebrandt and Suttie, 1982). Fasco and co-workers (1982) have demonstrated that vitamin K quinone reductase is also sensitive to inhibition by 4-hydroxycoumarin anticoagulants.

Thus it is generally accepted that warfarin and related 4-hydroxycoumarin anticoagulants inhibit clotting factor synthesis by blocking the vitamin K-dependent γ-carboxylation of glutamic acid residues in precursors of clotting factors II, VII, IX and X (Jackson and Suttie, 1977). The warfarin receptor is thought to be associated with the enzyme vitamin K epoxide reductase and possibly with vitamin K quinone reductase. These enzymes are responsible for the regeneration of vitamin K_1 and the production of vitamin K_1 hydroquinone respectively, in the physiologically important vitamin K-epoxide cycle (Bell and Matschiner, 1972).

Under normal circumstances, the vitamin K-dependent clotting factors II, VII, IX and X and the anticoagulant proteins C and S, are present in the plasma at steady state concentrations because they are synthesised at a given rate and degraded at a rate proportional to their concentration. When clotting factor synthesis is inhibited by anticoagulant therapy, the clotting factors and anticoagulant proteins disappear from plasma by a process which displays monoexponential decline. The rate of decay is determined by the half-life of the individual vitamin K-dependent proteins (Table 1.2). Thus factor VII and protein C disappear rapidly and prothrombin (factor II) slowly.

Several studies have investigated the relative potencies of the enantiomers of warfarin in man (Breckenridge et al., 1974; O'Reilly, 1974a; Wingard et al., 1978). Despite its shorter half-life, S(-) warfarin has been demonstrated to be approximately three times more

potent than R(+) warfarin at inducing hypoprothrombinaemia. This illustrates the greater receptor sensitivity to S(-) warfarin than to R(+) warfarin.

1.3.3 Pharmacokinetic - Pharmacodynamic Models for Warfarin

It may take some time for the pharmacological response to a drug to reflect the plasma concentrations of that drug. This delay may be due to the time required for equilibrium to occur between plasma and the site of action of the drug. For example, the administration of digoxin for the treatment of congestive heart failure. Alternatively, it may arise when the response monitored is an indirect measure of drug effect. This is true for the time delay observed following warfarin administration (Figure 1.12). The direct effect of warfarin is the inhibition of the synthesis of the vitamin K- dependent clotting factors II, VII, IX and X. However, warfarin does not exert an immediate effect because this compound only affects clotting factor synthesis and not clotting factor activity. Therefore the rate of change in clotting factor activity is determined partly by clotting factor half-life (Table 1.2) and as a consequence the maximum effect of a dose of warfarin is not seen until 1 to 2 days after administration.

Thus, warfarin is an indirectly acting anticoagulant and this must be taken into account to describe the extent and the time course of changes in clotting factor concentrations, collectively known as prothrombin complex activity (P.C.A.). This factor is called the physiological effect relationship (Holford and Sheiner, 1981).

It is important to determine the relationship between plasma concentration and the direct effect of warfarin, in order to distinguish between changes in the pharmacokinetics and the changes in the pharmaco-

FIGURE 1.12 The delay in pharmacological response following warfarin administration.
(From Nagashima et al., 1969)

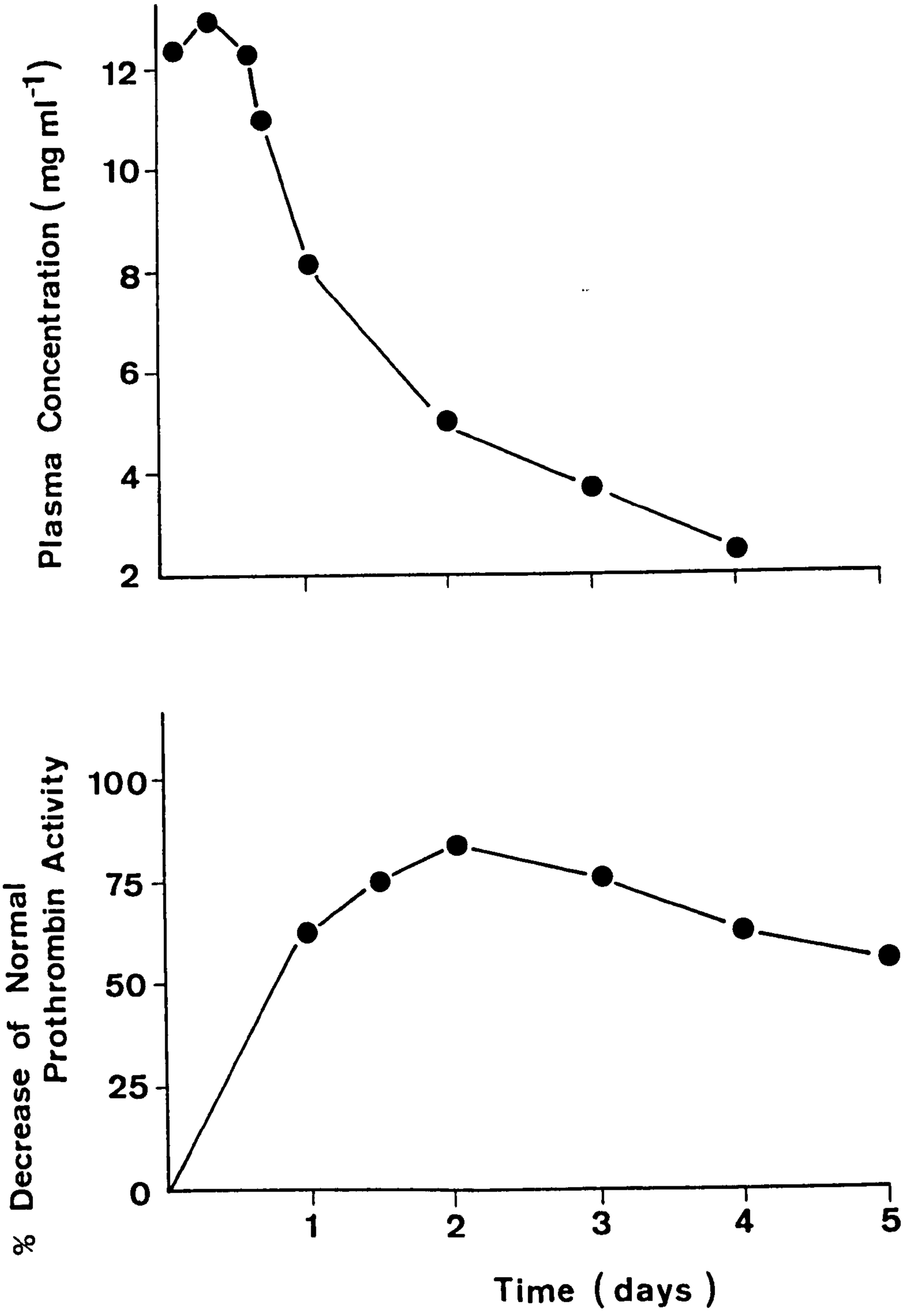


TABLE 1.2 The half-lives of vitamin K-dependent clotting factors

Factor	Half-life (h)
II	60
VII	6
IX	14
X	40
Protein C	6 - 8

dynamics of warfarin. The most commonly applied model for relating warfarin plasma concentration to the inhibition of synthesis of the prothrombin complex, is the log linear-model (Nagashima et al., 1969; Shepherd et al., 1979; Yacobi et al., 1980). This illustrates a linear relationship between the log of drug concentration in plasma at any given time and the pharmacological effect at that time. Shepherd and co-workers (1979) successfully used this model to compare the pharmacodynamics of warfarin in young and elderly patients. They demonstrated that the rate of prothrombin complex elimination was reduced by 50% in the older group, but the concentration of warfarin required to reduce synthesis by 50%, and thus achieve the same steady-state prothrombin complex concentration was the same in both groups. Furthermore this log-linear model has been shown to be useful in elucidating the mechanism of the increased hypoprothrombinaemic effect of warfarin by phenylbutazone in rats. Yacobi and co-workers (1980) determined that phenylbutazone decreases warfarin elimination and also displaces it from plasma proteins. Using total warfarin concentration to describe the effect on prothrombin complex synthesis, phenylbutazone appeared to potentiate the effect of warfarin by a factor of 30, however when unbound concentrations were used the potentiation was only three fold.

Other models may also be used to describe the pharmacological effect of warfarin. O'Reilly (1974a) used a linear model in which effect, as determined by change in prothrombin time from a control value, is proportional to plasma concentration to investigate the relative potencies of the enantiomers of warfarin in a group of individuals. He avoided the need to describe the complex relationship between plasma warfarin concentration, time and anticoagulant effect by relating the

area under the effect - time curve to the corresponding area under the concentration-time curve. It was then possible to show a linear relationship between these areas in this group of individuals. Thus O'Reilly used the linear model but included a value for the area under the effect - time curve when no drug was present which, by definition, is zero.

In contrast, in an investigation of the pharmacokinetics and the pharmacodynamics of the enantiomers of warfarin, Breckenridge and co-workers (1974) employed the linear model but with a fixed baseline of effect. That is a specific degree of anticoagulation as determined by % control activity in all patients.

Both examples of the linear model display important disadvantages. As a consequence of the use of the linear model with a value for the area under the effect - time curve when no drug was present (O'Reilly, 1974a), the calculated potency of S(-) warfarin was lower than it was using a fixed baseline of effect (Breckenridge et al., 1974). However, the fixed baseline linear model poses the practical problem of the maintenance of a specific degree of anticoagulation throughout the study.

The log-linear model appears to be the most suitable model to use, however it is now considered ethically difficult to obtain values for the rate constant of degradation (K_d) in man, and therefore those values obtained by Nagashima and co-workers (1969) have to be used. In experimental animals a value for K_d can be determined for each animal.

1.3.4 Clinical Aspects of Warfarin Therapy

In complete contrast to patients with defects of blood coagulation such as haemophilia, where fatal haemorrhage may result from a

lack of blood clotting following injury, many individuals suffer from disorders of normal haemostasis, in which intravascular clotting with the formation of thrombi or emboli occurs, resulting in the blockage of blood vessels. A thrombus is a clot of blood which is fixed to the wall of a blood vessel. Thrombi tend to form at sites of damage in the vascular endothelium. In veins, thrombus formation is commonly associated with inflammation (phlebitis), whilst in arteries, thrombi tend to form atheromatous plaques. An embolus is a fragment of a clot broken off from a thrombus. Emboli are carried in the blood stream until they lodge in an artery and cause a blockage. Thrombi and emboli lead to conditions including venous thrombosis, pulmonary embolism, stroke and myocardial infarction.

Blood clots in both arteries and veins are composed not only of fibrin but also of platelets. Venous thrombi are composed largely of fibrin with a large number of red blood cells and some platelets, whereas arterial thrombi are primarily composed of platelets with some fibrin and red blood cells. As a consequence of the different composition of venous and arterial thrombi, different therapeutic approaches are employed in the treatment of these conditions. Nevertheless, it is still not known whether disorders of platelet activation, or inappropriate activation of the clotting cascade lead to thrombo-embolic disorders.

Since the successful clinical trials of the early 1940s, coumarin anticoagulants have been used in the treatment of venous thrombosis. However, the use of these compounds in the treatment of arterial thrombosis has been a matter of debate. The major problem of the use of warfarin and related 4-hydroxycoumarin anticoagulants for arterial thrombosis is the fact that the gap between efficient therapeutic

control and an unacceptable risk of bleeding is extremely narrow, whereas in venous thrombosis, it is considerably wider.

Although Vries and co-workers (1980) have thoroughly investigated ways in which patients can be maintained within the narrow, but clearly defined therapeutic range for arterial thrombosis, clinicians are still reluctant to use long term anticoagulant therapy for patients with ischaemic heart disease (Drug Ther. Bull. 1982). In contrast, but despite the lack of controlled clinical trials, strong evidence suggests that permanent treatment with 4-hydroxycoumarin anticoagulants reduces morbidity from embolism in patients with atrial fibrillation due to mitral stenosis (Fleming and Bailey, 1971). Similarly long term anticoagulant therapy is believed to reduce the risk of embolism in patients with atrial fibrillation due to chronic sinoatrial disease (Fairfax et al., 1976), thyrotoxicosis (Staffurth et al., 1977) or congestive cardiomyopathy (Rogers and Sherry, 1976).

In the treatment of both venous and arterial thrombosis, the improvement of laboratory control of the degree of anticoagulation, and a better understanding of the nature of coumarin anticoagulants, have led to more beneficial dosage regimens. Table 1.3 illustrates the present commonly accepted indications for oral anticoagulant therapy.

In the treatment of both venous and arterial thrombi there is a relatively narrow therapeutic window when compared to many other drugs. The fine balance between under anticoagulation, in which clotting factor synthesis is not inhibited to an extent sufficient to prevent thrombus formation, and over anticoagulation which brings with it the risk of haemorrhage, means that patients on oral anticoagulant therapy require regular monitoring of their degree of anticoagulation.

TABLE 1.3 Commonly accepted indications for oral anticoagulant treatment.

1. Venous thrombosis and pulmonary embolism

2. Myocardial infarction

3. Valvular heart disease, prosthetic valves, cardiac arrhythmia and cardiomyopathy

4. Systemic embolism

5. Transient cerebral ischaemia

6. Reconstructive artery surgery and atherosclerotic vascular disease.

The prothrombin time,which was introduced in 1935 by Quick and co-workers is still the most widely used determinant of anticoagulation in clinical practice. Basically the test involves the collection of blood into a solution of trisodium citrate. This removes calcium by the formation of the insoluble calcium citrate. The plasma is then separated and a thromboplasin preparation is added. The calcium concentration is restored to the optimal level and the clotting time is measured. Clotting time is dependent on the availability of clotting factors II, VII and X, and thus reflects the activity of the extrinsic and common pathways of the clotting cascade.

The regular monitoring of the degree of anticoagulation in patients taking warfarin becomes even more important when other drugs are introduced into the patients daily therapeutic regimen. Warfarin is liable to drug interactions which may either increase or decrease its anticoagulant effect, and as a consequence of the narrow therapeutic window of warfarin, these effects may be life threatening.

Cholestyramine has been shown to reduce the absorption of warfarin and thus reduce its pharmacological effect (Robinson et al., 1971; O'Reilly, 1974b). An increase in the hypoprothrombinaemic effect of warfarin is seen with drugs such as phenylbutazone (Aggeler et al., 1967; Koch-Weser and Sellers, 1971) which displace warfarin from plasma proteins. Inhibition of the metabolism of warfarin by drugs including metronidazole (Kazmier, 1976), cotrimoxazole (O'Reilly and Motley, 1976) and cimetidine (Serlin et al., 1979) leads to an increased anticoagulant effect of warfarin. In contrast, drugs which induce the enzymes of the mixed function oxidase system, such as barbiturates (Corn, 1966) reduce the hypoprothrombinaemic effect of warfarin.

Disease and age also affect the pharmacological effect of warfarin. Liver disease (Brodie et al., 1959) and thyrotoxicosis (Self et al., 1976) are known to potentiate the response to warfarin. Similarly, the anticoagulant response to warfarin is greater in elderly patients than in young patients (O'Malley et al., 1977).

When the prothrombin time test reveals that an individual is over-anticoagulated either due to increased responsiveness in the clinical situation or coumarin overdose, the current therapeutic advice is to administer vitamin K_1, initially intravenously but further vitamin K_1 may be given orally (British National Formulary). Administration of the vitamin continues ad hoc until the prothrombin time returns to normal.

However, there is little information relating the pharmacokinetics of vitamin K_1 to its pharmacodynamics. Experiments in chronically anticoagulated rabbits have illustrated that plasma concentrations of the vitamin of the order of 1 $\mu g\,ml^{-1}$ are required to drive clotting factor synthesis (Park et al., 1984). As a consequence of the rapid elimination of vitamin K_1 from both man and experimental animals, frequent administration of the vitamin is required to maintain such high plasma concentrations. This observation has been made in individuals poisoned with 4-hydroxycoumarin anticoagulants (Barlow et al., 1982).

1.4 Aims of the Present Work

An individual's pharmacological response to a dose of warfarin is dependent on both the pharmacokinetics and the pharmacodynamics of that drug. Thus the ability to be able to distinguish between these two processes is of utmost importance to improve our understanding of the pharmacology of warfarin.

The initial aim of the studies described in this thesis was to develop an animal model which could be employed to evaluate the relative contributions of the pharmacokinetics and the pharmacodynamics of any 4-hydroxycoumarin anticoagulant to the overall pharmacological effect, and then to demonstrate the application of this method to the determination of the mechanisms responsible for clinically relevant interactions with warfarin.

An important problem experienced by clinicians when initiating warfarin therapy, is choice of dose. The aim of the present work was to investigate the use of a novel parameter, the plasma concentration of vitamin K_1 2,3-epoxide, in conjunction with the pharmacokinetics and the pharmacodynamics of warfarin, to determine the pharmacological effect of warfarin and its enantiomers in well controlled groups of volunteers and large groups of patients.

The final aim of the studies described in this thesis was to investigate the relationship between the pharmacokinetics and the pharmacodynamics of vitamin K_1 in both man and experimental animals, with a view to a better understanding of the antagonism of vitamin K_1 by warfarin in patients on anticoagulant therapy and in the situation of chronic coumarin anticoagulation experienced in individuals poisoned with the novel 4-hydroxycoumarin anticoagulants brodifacoum and difenacoum.

CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS

2.2 EXPERIMENTAL ANIMALS

2.2.1 Rabbits

2.2.2 Rats

2.3 ANALYTICAL TECHNIQUES

2.3.1 Determination of Warfarin Concentrations in Plasma by High Performance Liquid Chromatography.

2.3.2 General Precautions in Handling Vitamin K_1.

2.3.3 Determination of Vitamin K_1 and Vitamin K_1 2,3-Epoxide in Plasma by High Performance Liquid Chromatography with Ultra-Violet Detection.

2.3.4 Determination of Vitamin K_1 and Vitamin K_1 2,3-Epoxide in Rat Liver by High Performance Liquid Chromatography with Ultra-Violet Detection.

2.3.5 Determination of Vitamin K_1 in Plasma by High Performance Liquid Chromatography with Electrochemical Detection.

2.3.6 Determination of Vitamin K_1 in Liver by High Performance Liquid Chromatography with Electrochemical Detection.

2.3.7 Determination of Prothrombin Time for Rat, Rabbit and Human Plasma.

2.3.8 Determination of the Effect of Cimetidine on the Plasma Protein Binding of Warfarin *in vitro*.

2.4 PHARMACOKINETIC ANALYSIS OF RESULTS

2.1 CHEMICALS

Racemic, R(+) and S(-) warfarin were obtained from Ward Blenkinsop, Widnes. Racemic [^{14}C]-warfarin was obtained from the Radiochemical Centre, Amersham. Brodifacoum was a gift from Sorex Laboratories, Widnes. Vitamin K_1 was obtained from Sigma Chemicals, Poole. Vitamin K_1 (Konakion$^{(R)}$) and 2-methyl-3-geranyl-geranyl-1,4-naphthoquinone (MK4) were gifts from Hoffmann-La Roche, Welwyn Garden City. 2-methyl-3-farnesyl-farnesyl-1,4-naphthoquinone (MK6) was a gift from Dr. M.J. Shearer, Guy's Hospital, London. 2-chloro-3-phytyl-1,4-naphthoquinone (Cl-K) was a gift from Sorex Laboratories, Widnes. Vitamin K_1 2,3-epoxide was synthesized by the method of Tischler et al., (1940), the structure confirmed by UV absorbance between 200 and 400 nm and its purity verified by reversed-phase partition TLC (Shearer et al., 1977) and the normal-phase HPLC system described in section 2.3.3. No residual vitamin K_1 was detected. Cimetidine was obtained from Smith, Kline and French, Welwyn Garden City. Manchester Comparative Thromboplastin was obtained from the National (UK) Reference Laboratory for Anticoagulant Reagents and Control, Manchester. All general reagents were obtained from BDH, Poole. The acetone used was from Koch-Light Laboratories, Colnbrook, and all other solvents were HPLC grade from Fisons, Loughborough.

2.2 EXPERIMENTAL ANIMALS

2.2.1 Rabbits

Male New Zealand White rabbits (2.4 to 3.0 kg) were used in this work and were obtained from Bantin and Kingman, Poole. The rabbits had free access to food and water throughout the experiments and were maintained on Diet R14 obtained from Labsure Animal Foods, Poole. The average daily intake of vitamin K_1 was calculated to be approximately 60 $\mu g\ kg^{-1}$.

2.2.2 Rats

Male Wistar warfarin-sensitive rats (200 to 300g) were used in this work and were obtained from Bantin and Kingman, Poole. Before use the rats were caged in groups of 6 in well ventilated rooms at 21^{o}C with a 12h light cycle, and had free access to food and water. The rats were maintained on Diet 41B obtained from Labsure Animal Foods, Poole. The average daily intake of vitamin K_1 was calculated to be approximately 40 $\mu g\ kg^{-1}$.

2.3 ANALYTICAL TECHNIQUES

High Performance Liquid Chromatography

It is generally accepted that HPLC first appeared as a separation technique during the period of 1964 to 1965 (White, 1984). Since that time, and especially over the last decade HPLC has become one of the most important and widely used methods for the determination of drug concentration in biological material. It is a technique which displays a high degree of selectivity, accuracy, sensitivity and specificity. These factors contribute to its ability to simultaneously discriminate for and analyse closely related drugs and their metabolites. However, like all analytical techniques it has shortcomings. For example, samples require a clean up step before they can be analysed, and analysis of many samples cannot be carried out simultaneously in contrast with many radioimmunoassays.

Nevertheless, HPLC was found to be the ideal choice of analytical technique for the determination of warfarin, vitamin K_1 and its metabolite, vitamin K_1 2,3-epoxide in the biolgical samples obtained in this work. Both normal-phase and reversed-phase HPLC systems have been utilized, which use a non-polar support with a polar mobile phase and a polar stationary phase with a non-polar eluent respectively.

Instrumentation

Two HPLC systems were used in these studies. The first comprised of an Altex 110A solvent delivery pump, a Gilson N1 potentiometric recorder and an Altex 160 fixed wavelength detector fitted with either a 254 nm or 313 nm filter. The second system comprised of a Spectra Physics SP 8700 solvent delivery system, a Philips PM

8251 single pen chart recorder and either a Kratos Spectroflow 773 variable wavelength detector set at 254 nm or an ESA Coulochem S100A detector linked to an ESA 5021 conditioning cell plus an ESA 5011 analytical cell. Both systems were fitted with Rheodyne 7125 injection valves and were run at ambient temperature. All chromatography columns were supplied by either HPLC technology Ltd. or Technicol, Ltd. and were repacked when column efficiency was seen to decline. All solvents used for chromatography were degassed by sonication prior to use unless otherwise stated.

2.3.1 Determination of Warfarin Concentrations in Plasma by High Performance Liquid Chromatography

Since the 1950s warfarin has been widely used for the prophylaxis and the treatment of thrombo-embolic disease, and as a consequence many analytical methods have been developed with which to quantify this 4-hydroxycoumarin anticoagulant in plasma and other biological material. A spectrophotometric method developed by O'Reilly and co-workers (1963) for the measurement of warfarin in plasma has been used extensively to study the pharmacokinetics of this anticoagulant compound (Nagashima et al., 1969; Levy et al., 1974). However, this method could only be used following relatively large doses of warfarin since the limit of sensitivity of this assay was approximately 1 μg ml^{-1}. Similarly a fluorimetric method (Corn and Berberich, 1967) has been employed to study the pharmacokinetics of the enantiomers of warfarin after single oral doses of 15 to 100 mg (Hewick and McEwen, 1973). Fluorimetric and spectrophotometric methods cannot distinguish between parent drug and metabolites, thus thin layer chromatography (TLC) has been used in conjunction with these detection methods to determine or to improve assay specificity (Lewis et al., 1970). Gas liquid chromatography (GLC) has been used to determine relatively low concentrations (250 ng ml^{-1}) of warfarin in plasma (Kaiser and Martin, 1974). This method requires derivatization of the warfarin molecule with an electron capturing group and subsequent GLC electron capture detection.

At present the most common method for the quantification of warfarin in biological material is HPLC. As a moderately polar compound with both UV and fluorescent properties, warfarin is well suited to analysis by HPLC and has been successfully assayed in plasma by both normal-phase (Shearer, 1986) and reversed-phase methods

(Hackett et al., 1985). The differences in specificity between the fluorimetric method of Corn and Berberich (1967) and an HPLC technique with UV detection was demonstrated by Vesell and Shively (1974). For a group of 12 individuals the mean plasma half-life of warfarin was determined to be 55.9 h by the fluorimetric method and 36.3 h by the HPLC method. Most studies report mean half-lives of 35 to 45 h. Furthermore, plasma concentrations of warfarin in the range of 50 to 100 ng ml^{-1} can be determined using HPLC, thus illustrating the sensitivity of this technique.

The studies described in this thesis employed a slightly modified version of an HPLC method described by Shearer (1986) to determine the concentrations of warfarin in both rabbit and human plasma. This method involves micro-extraction of samples, which facilitates the rapid analysis of small volumes of plasma. It also allows direct injection of the extracting solvent, methyl-t-butyl ether onto the analytical column without any pre-column clean up of the extract.

Method

Standard solutions of racemic, R(+) and S(-) warfarin and the internal standard acenocoumarol were prepared by dissolving solid compound (10 mg) in methyl-t-butyl ether (3 ml) and dichloromethane (2 ml). This gave a final stock solution of 2 mg ml^{-1} which was further diluted with methyl-t-butyl ether to give working standards of 1,10 and 100 µg ml^{-1}.

Following the addition of acenocoumarol, warfarin was extracted from duplicate plasma samples (1 vol; 100 to 400 µl) with methyl-t-butyl ether (2 vol) by vortexing (2 min) after protein precipitation with hydrochloric acid (5M; 0.5 vol). An aliquot (100 µl) of the

organic extract was injected directly onto the HPLC system. The analytical column (25 cm x 4.5 mm i.d.) was packed with Spherisorb 5 µm nitrile and was protected by a guard column (2.5 cm x 4.5 mm i.d.) packed with Partisil 10 silica. The mobile phase consisted of hexane-isopropylalcohol-dichloromethane - acetic acid (85:10:5:1 v/v) at a flow rate of 2.2 ml min^{-1}. Warfarin was detected by UV absorbance at 313 nm, with a sensitivity of up to 0.002 a.u.f.s.

The extraction efficiency for warfarin (1 µg ml^{-1}) from rabbit plasma was 95 $\pm$ 3% (90 to 98%, n = 6) and from human plasma was 92 $\pm$ 5% (84 to 98%, n = 6). The retention times were 5.8 min and 9.4 min for warfarin and acenocoumarol respectively (Figure 2.1a). Plasma warfarin concentrations were determined by the ratio of warfarin peak height to the peak height of the internal standard. The linear regression lines obtained from the standard curves for racemic, R(+) and S(-) warfarin in both rabbit and human plasma can be seen in Table 2.1. Intra-assay variation was calculated by the repeated (n = 14) analysis of aliquots (200 µl) of a single spiked plasma sample and gave a coefficient of variation of 2.8%. Interassay variation was calculated by the analysis of aliquots (200 µl) of a single spiked plasma sample on separate days (n = 14), over a period of several weeks and gave a coefficient of variation of 3.9%. The limit of sensitivity of this assay for the determination of warfarin in rabbit and human plasma was 50 ng ml^{-1}. An experimental chromatogram from this method is illustrated in Figure 2.1b.

FIGURE 2.1 Determination of warfarin in rabbit plasma

(a) separation of 1000 ng of warfarin and acenocoumarol.

(b) extract of rabbit plasma (200 µl) taken 4 h after intravenous administration of warfarin (6.3 mg kg^{-1}).

Peaks; 1 = warfarin

2 = acenocoumarol (internal standard)

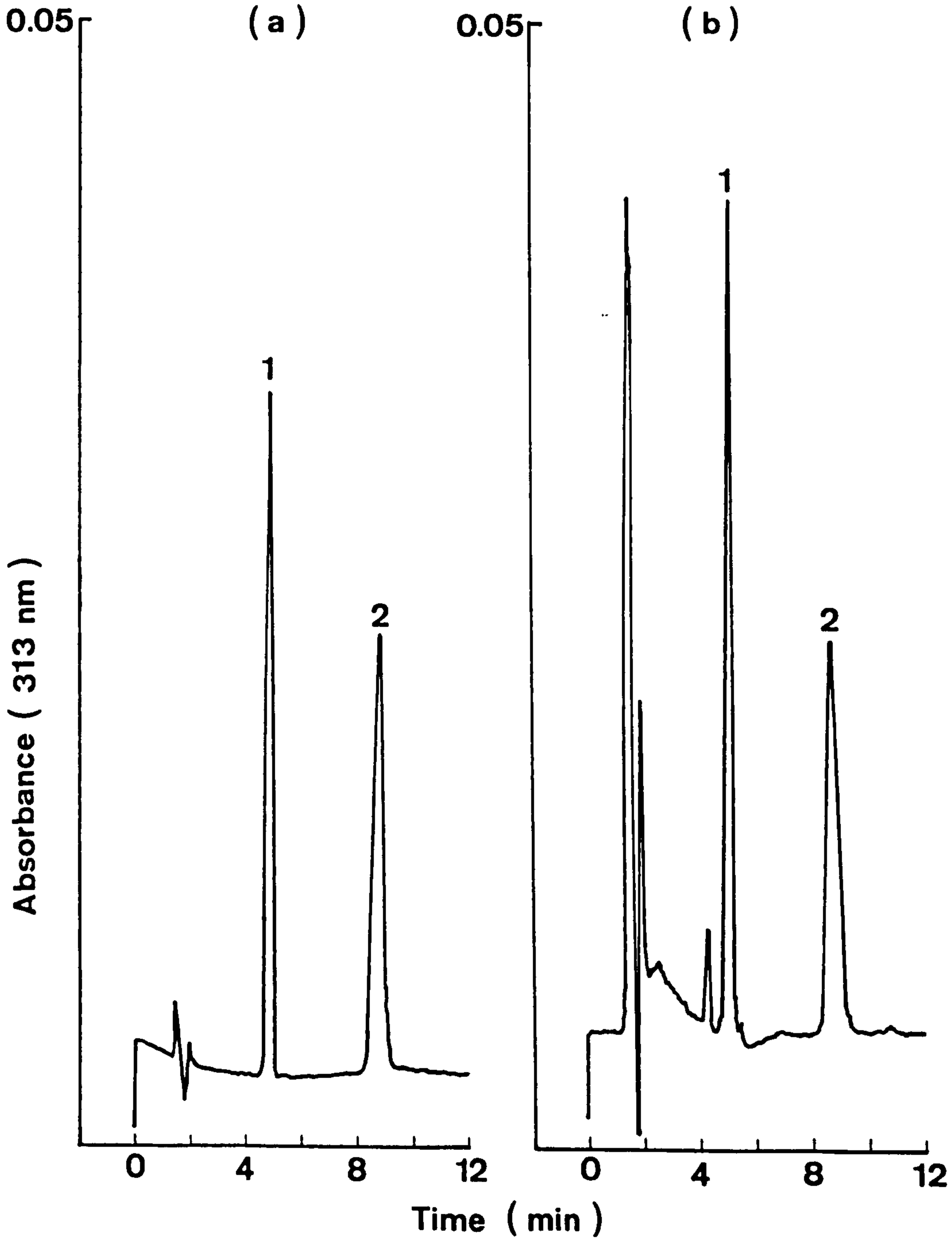


TABLE 2.1 The linear regression analysis of the standard curves obtained for the warfarin assay in rabbit and human plasma.

	Rabbit Plasma		
	r	c	m
Racemic warfarin	0.998	0.057	1.224
R(+) Warfarin	0.998	0.049	1.081
S(-) Warfarin	0.998	0.018	1.269
	Human Plasma		
	r	c	m
Racemic Warfarin	0.995	0.036	1.233
R(+) Warfarin	0.997	0.065	1.507
S(-) Warfarin	0.992	0.058	1.247

Each value represents the mean of four standard curves

r = correlation coefficient

c = intercept on y axis

m = slope of line

2.3.2 General Precautions in Handling Vitamin K_1

Throughout the course of the studies described in this thesis many difficulties arose due to the physical properties of vitamin K_1. Most important is the light sensitive nature of the vitamin. Photolysis results in the conversion of vitamin K_1 to a complex mixture of products. The major product having been identified as vitamin K_1 hydroperoxide (Canfield et al., 1985). Furthermore the conversion of the active *trans*-vitamin K_1 to the inactive *cis*-vitamin K_1 is known to be enhanced by fluorescent light. To overcome this problem all containers used to store vitamin K_1 or vitamin K_1 solutions were made from amber glass or were covered in aluminium foil. Throughout any extraction procedure involving vitamin K_1, the samples were protected at all times from fluorescent light in tubes covered with aluminium foil. Whenever possible daylight was preferred to artificial (fluorescent) lighting in the laboratory.

To prevent the binding of vitamin K_1 to the surface of glass containers, all glassware was rinsed with 5% dimethyldichlorosilane in toluene, which binds to active sites on the glass. As a consequence of its lipophilic nature, vitamin K_1 was never stored in plastic containers.

Several organic solvents were found to be incompatible with vitamin K_1. For example, if a solution of vitamin K_1 in methanol was evaporated to dryness under a stream of oxygen free nitrogen, a considerable loss (90%) of vitamin K_1 was experienced. Similarly the evaporation of a solution of vitamin K_1 in dichloromethane resulted in the loss of 25% of the vitamin. Purification of both these solvents by distillation resulted in a better recovery of vitamin K_1 (methanol, 50%; dichloromethane, 85%). In addition vortex

evaporation further increased the recovery of vitamin K_1 from many organic solvent solutions including methanol, dichloromethane, hexane and ethanol.

Standard solutions of vitamin K_1 in hexane could be used for at least 4 weeks before concentrations were seen to decline. However standard solutions of vitamin K_1 made up in acetonitrile or ethanol had to be replaced every 2 weeks.

2.3.3 Determination of Vitamin K_1 and Vitamin K_1 2,3-Epoxide in Plasma by High Performance Liquid Chromatography with Ultra-Violet Detection

The synthesis of radiolabelled vitamin K_1 by Matschiner (1970) unleashed a novel method with which to study vitamin K_1 and its antagonism by 4-hydroxycoumarin anticoagulants. As a consequence, throughout the 1970s vitamin K_1 disposition and metabolism was studied almost exclusively using [^{3}H] vitamin K_1 (Matschiner and Bell, 1972; Shearer et al., 1977). Following a tracer dose of radiolabelled vitamin K_1 many workers used TLC to separate the *cis* and *trans* isomers of vitamin K_1 (Knauer et al., 1975) and also to separate vitamin K_1 from its metabolite vitamin K_1 2,3-epoxide (Trenk et al., 1980; Park et al., 1979), thus enhancing the specificity of their assays. However, with the introduction of HPLC as a routine analytical technique with which to study the K vitamins, TLC was effectively made redundant. The major reasons for this include the higher resolution and greater sensitivity of HPLC when compared to TLC. A report comparing the two methods of detection (Donnahey et al., 1979) stated that the resolving power of HPLC is superior to that of TLC to the extent that to reach a comparable level of separation to that by HPLC would require at least two successive TLC systems. These workers also reported the detection of 5 to 10 ng of vitamin K_1 using HPLC but only 250 to 500 ng by TLC, thus illustrating the greater sensitivity of HPLC. Furthermore, HPLC is a much more rapid technique than TLC, making it ideally suited to the routine analysis of biological samples.

Vitamin K_1, like the other K vitamins has two outstanding physical attributes which make it an ideal compound for analysis by HPLC. It is highly lipophilic and neutral, thus making it compatible with a

wide range of organic solvents which may be present in the mobile phase of the HPLC system. In addition vitamin K_1 absorbs light in the ultraviolet region of the electromagnetic spectrum which enables sensitive detection by photometric detectors. Both normal-phase (Shearer et al., 1979) and reversed-phase (Wilson and Park, 1983) HPLC systems have been employed to quantify vitamin K_1 in biological material. Reversed-phase methods appear to be more suitable for the separation of the K vitamins, whereas the application of normal phase assays allows resolution of the isomers of vitamin K_1 (Haroon et al., 1981).

The method used in the studies described in this thesis was the normal-phase assay reported by Wilson and Park (1983). This method was found to be specific, rapid and reproducible when employed to determine concentrations of vitamin K_1 and its metabolite vitamin K_1 2,3-epoxide in rabbit, rat and human plasma after the administration of a pharmacological dose of the vitamin.

Method

Standard solutions of vitamin K_1, vitamin K_1 2,3-epoxide and the internal standard, MK4 were prepared in hexane at concentrations of 5, 50 and 500 μg ml^{-1} and were stored at room temperature.

All plasma samples were analysed in duplicate. An aliquot of internal standard solution (200 to 1000ng) was put into a glass tube prior to the addition of plasma (1 vol; 0.5 to 1.0 ml). The contents of the tube were vortexed (30 s) and left at room temperature to equilibrate (15 min). Methanol was then added (1 vol; 0.5 to 1.0 ml) to precipitate plasma proteins immediately followed by the addition of hexane (5 ml). The extraction was carried out by gentle mechanical

tumbling (30 r.p.m.; 15 min). To ensure complete separation of the methanol-water phase from hexane, the tube was centrifuged (2000 x g; 2 min). The organic layer was then carefully removed, transferred to a clean tube and evaporated to dryness under vacuum at 50°C. The extract residue was redissolved in hexane (60 to 100 µl) and an aliquot (20 µl) was injected onto the HPLC system for analysis. The analytical column (25 cm x 4.5 mm i.d.) was packed with Partisil 10 silica and was protected by a guard column (2.5 cm x 4.5 mm i.d.) containing the same stationary phase. The mobile phase was 0.23% acetonitrile in hexane at a flow rate of 2 ml min^{-1}. Vitamin K_1 and vitamin K_1 2,3-epoxide were detected by UV absorbance at 254 nm, with a sensitivity of up to 0.002 a.u.f.s.

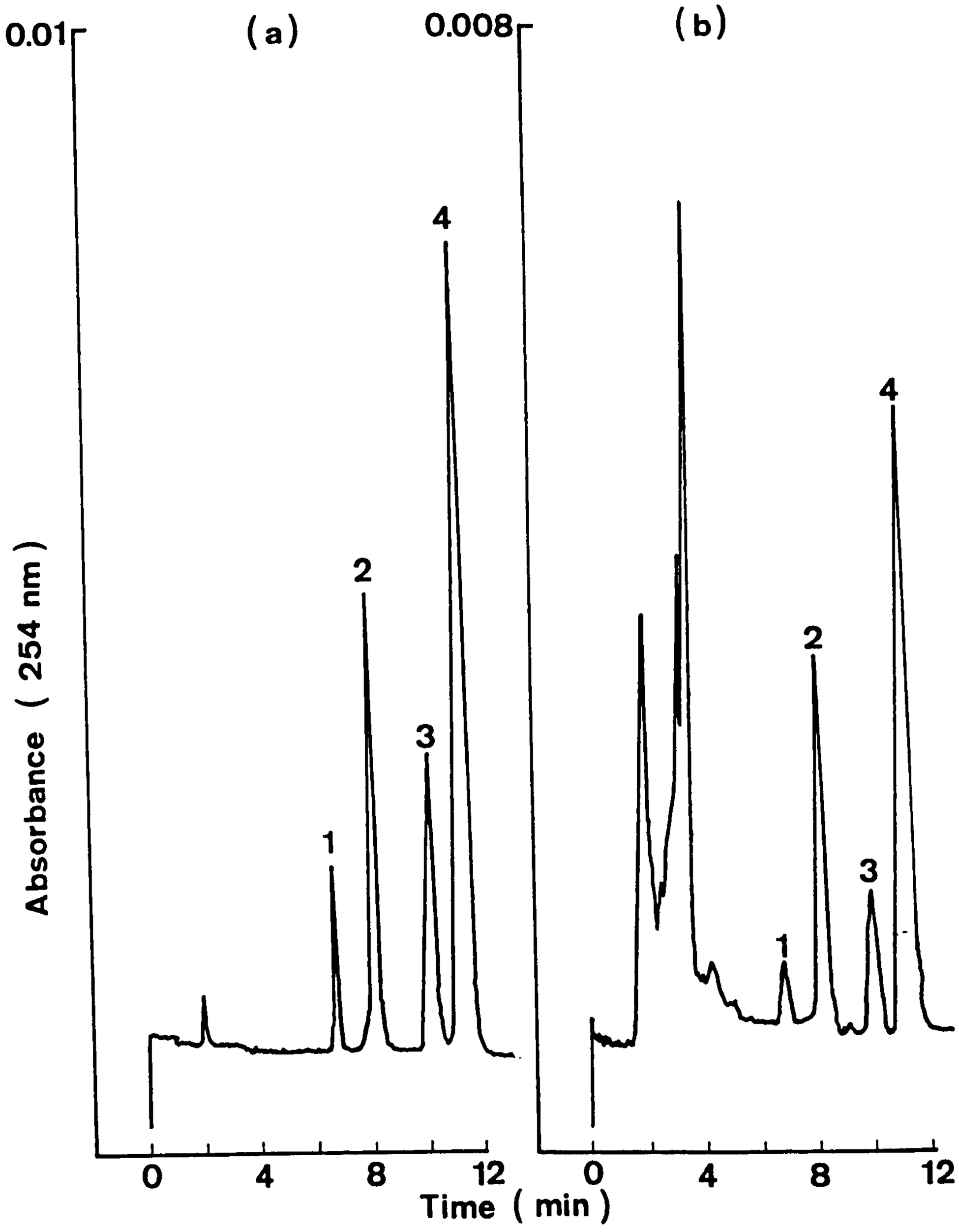
The extraction efficiency for vitamin K_1, vitamin K_1 2,3-epoxide and MK4 (0.5 µg ml^{-1}) from rat, rabbit and human plasma were all repeatedly (n = 6) greater than 90%. Retention times for *cis*-vitamin K_1, *trans*-vitamin K_1, vitamin K_1 2,3-epoxide and MK4 were 7.2, 8.0, 9.2 and 11.4 min respectively (Figure 2.2a). Plasma *cis*- and *trans*-vitamin K_1 and vitamin K_1 2,3-epoxide concentrations were measured by the ratio of *cis*- or *trans*-vitamin K_1 and vitamin K_1 2,3-epoxide peak heights to the peak height of internal standard, MK4. The mean linear regression data obtained from the standard curves for *cis*-vitamin K_1, *trans*-vitamin K_1 and vitamin K_1 2,3-epoxide in rat, human and rabbit plasma are shown in Table 2.2. All standard curves were constructed with an internal standard concentration of 400 ng ml^{-1}.

Intra-assay variation was calculated by the repeated (n = 6) analysis of aliquots (1 ml) of a single rat plasma sample containing 0.5 µg ml^{-1} of vitamin K_1 and vitamin K_1 2,3-epoxide and gave a coefficient of variation of 4.2 and 3.9% for total vitamin K_1 and vita-

FIGURE 2.2 Determination of vitamin K_1 and vitamin K_1 2,3-epoxide in rat plasma.

(a) separation of 100 ng of vitamin K_1, vitamin K_1 2,3-epoxide and MK4

(b) extract of rat plasma (1ml) taken 3h after intravenous administration of vitamin K_1 (1 mg kg^{-1}) and 9h after intraperitoneal administration of brodifacoum (10 mg kg^{-1}).



Peaks: 1 = *cis* - vitamin K_1 2 = *trans* - vitamin K_1

3 = vitamin K_1 2,3-epoxide

4 - MK4 (internal standard)

TABLE 2.2 The linear regression analysis of the standard curves obtained for the determination of vitamin K_1 and vitamin K_1 2,3-epoxide in plasma.

	Rat Plasma		
	r	c	m
Vitamin K_1 (*cis* + *trans*)	0.992	0.052	1.164
Vitamin K_1 2,3-epoxide	0.999	0.025	0.441
	Human Plasma		
	r	c	m
Vitamin K_1 (*cis* + *trans*)	0.998	0.033	1.366
Vitamin K_1 2,3-epoxide	0.999	0.008	0.556
	Rabbit Plasma		
	r	c	m
Vitamin K_1 (*cis* + *trans*)	0.999	0.019	1.456
Vitamin K_1 2,3-epoxide	0.999	0.062	0.628

Each value represents the mean of four standard curves.

r = correlation coefficient

c = intercept on y axis

m = slope of line

min K_1 2,3-epoxide respectively. Inter-assay variation was calculated by the analysis of aliquots (1 ml) of a single rat plasma sample containing 0.5 µg ml^{-1} of vitamin K_1 and vitamin K_1 2,3-epoxide on separate days (n = 6), over a period of several weeks and gave a coefficient of variation of 4.6 and 4.3% for total vitamin K_1 and vitamin K_1 2,3-epoxide respectively. The limit of sensitivity of the assay for *trans*-vitamin K_1, *cis*-vitamin K_1 and vitamin K_1 2,3-epoxide in rat, rabbit and human plasma was 30 ng ml^{-1}. The range of concentrations measured using this method of analysis was 30 to 136500 ng ml^{-1}. An experimental chromatogram for this method is shown in Figure 2.2b.

2.3.4 Determination of Vitamin K_1 and Vitamin K_1 2,3-Epoxide in Rat Liver by High Performance Liquid Chromatography with Ultra-Violet Detection.

Several workers have used radiolabelled vitamin K_1 to investigate the metabolism and the disposition of vitamin K_1 in the liver of both rabbits (Park et al., 1980) and rats (Trenk et al., 1980). However, this method required laborious extraction followed by reversed-phase partition TLC (Shearer et al., 1977). Consequently the aim of this work was to develop an HPLC assay to measure accurately vitamin K_1 and vitamin K_1 2,3-epoxide in the liver after intravenous administration of a pharmacological dose of vitamin K_1.

In a recently published method (Haroon et al., 1984) which utilized electrochemical detection to determine endogenous vitamin K_1 concentrations in rat liver, hexane was used to extract the hepatic vitamin. However, in my hands efficient extraction of vitamin K_1 could not be achieved by hexane alone. Acetone was required to extract vitamin K_1 and its epoxide from the lipophillic membranes present in liver homogenate. As a result, injection of the organic extract onto either a normal-phase or a reversed-phase system produced traces from which neither vitamin K_1 or vitamin K_1 2,3-epoxide could be quantified due to interference from other lipophillic material which co-extracted.

As a consequence, sequential chromatography was employed; the first step involved a normal-phase chromatographic separation originally developed to determine plasma concentrations of vitamin K_1 and vitamin K_1 2,3-epoxide (section 2.3.3). The second involved a reversed-phase system also developed to quantify vitamin K_1 and its epoxide

in plasma (Wilson and Park, 1983). However, the assay had to be slightly altered due to the nature of the extraction. Cl-K was used as the internal standard for this assay, as MK4 co-eluted with vitamin K_1 during reversed-phase chromatography.

The assay developed enabled the hepatic content of vitamin K_1 and vitamin K_1 2,3-epoxide in rat liver following a pharmacological dose of vitamin K_1 to be determined accurately and routinely.

Method

Standard solutions of vitamin K_1, vitamin K_1 2,3-epoxide and the internal standard, Cl-K were prepared in hexane at concentrations of 5, 50 and 500 μg ml^{-1} for normal-phase HPLC. The same concentrations of each compound were prepared in acetonitrile for reversed-phase HPLC. All standard solutions were stored at room temperature.

All liver homogenate samples were analysed in duplicate. To an aliquot of liver homogenate (100 μl), internal standard solution was added (400-1000 ng). The suspension was vortexed (30s) and left at room temperature to equilibrate (15 min). Acetone (0.5 ml) was then added and the mixture was mechanically shaken (20 min). Hexane (2 ml) was added, the contents of the tube shaken (10 min) and then centrifuged (5000 x g; 5 min) to ensure complete separation of the organic layer from the aqueous layer. The organic layer was removed, evaporated to dryness under vacuum at 50°C and then the extract residue was redissolved in normal-phase eluent (60 μl). Following injection of this solution (50 μl) onto the normal-phase HPLC system, the effluent was collected between 6 and 10 min to ensure complete recovery of vitamin K_1, vitamin K_1 2,3-epoxide and Cl-K (Figure 2.3b). Under vacuum at 50°C the effluent was evaporated to dryness and the

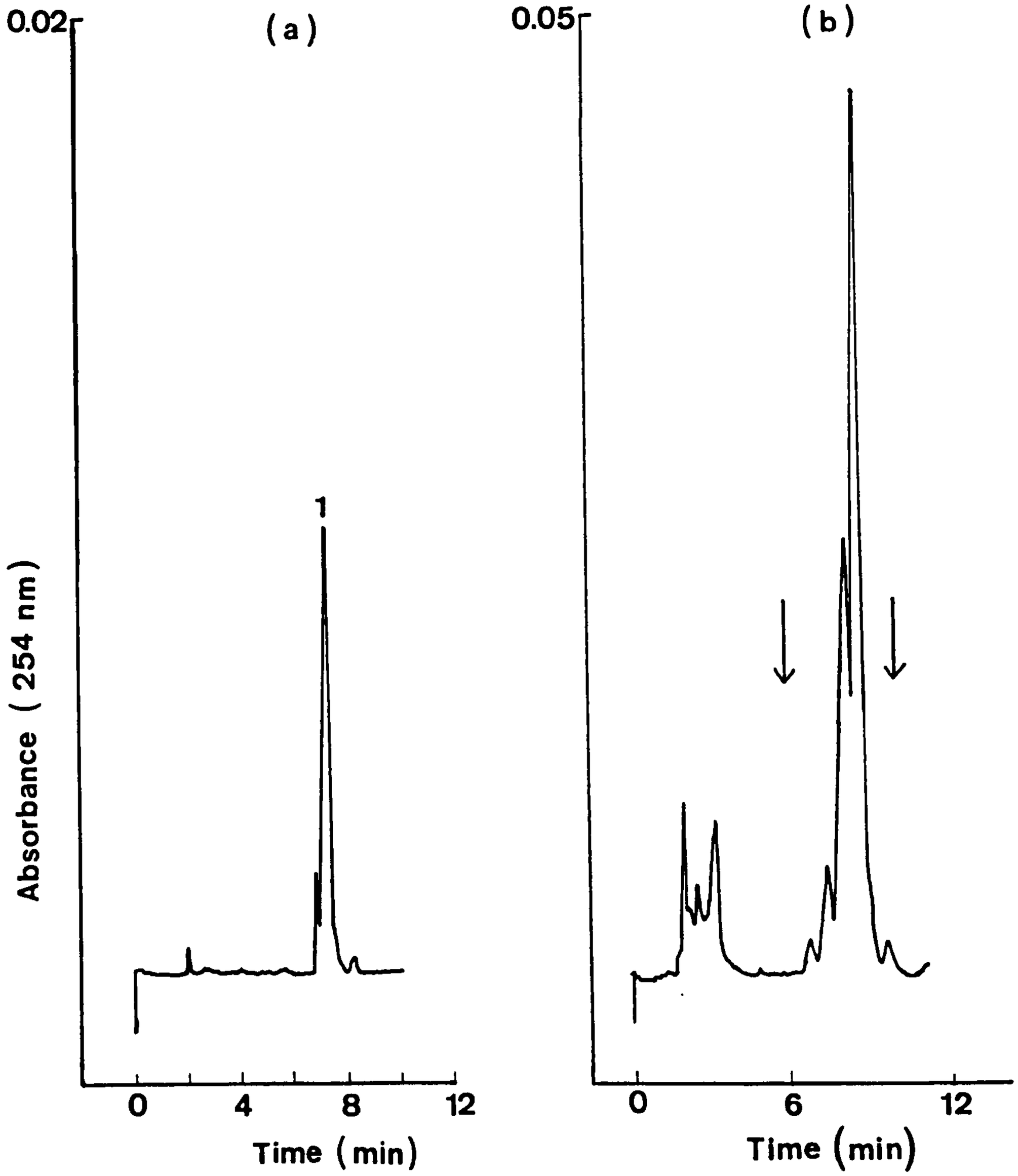
extract residue redissolved in reversed-phase eluent (100 µl); the solution (50 µl) was then injected onto the reversed-phase system for analysis. The normal-phase semi-preparative column was packed with Partisil 10 ODS (2.5 cm x 4.5 mm i.d.) and protected by a guard column (2.5 cm x 4.5 mm i.d.) packed with Partisil 10 silica gel. The mobile phase used was 0.23% acetonitrile in hexane at a flow rate of 2 ml min^{-1}. It had to be freshly made each day, thoroughly mixed and degassed by sonication immediately prior to use. Vitamin K_1 and vitamin K_1 2,3-epoxide were detected by UV absorbance at 254 nm, with a sensitivity of up to 0.002 a.u.f.s.

The reversed-phase analytical column was packed with Ultrasphere ODS C_{18} (2.5 cm x 4.5 mm i.d.) and protected by a guard column (2.5 cm x 4.5 mm i.d.) packed with Ultrasphere ODS C_{18}. The mobile phase used was 12.5% dichloromethane in acetonitrile at a flow rate of 1 ml min^{-1}. A continual stream of helium passing through the solvent ensured thorough degassing. UV detection was effected at 254 nm with a sensitivity of up to 0.002 a.u.f.s.

The final overall recoveries of vitamin K_1, vitamin K_1 2,3-epoxide and Cl-K from rat liver homogenate were 61, 77 and 50% respectively, at a liver concentration of 0.5 g ml^{-1}. The range of concentrations measured using this assay were 0.25 to 100µg g^{-1} with a limit of sensitivity of 250 ng g^{-1}.

Using the semi-preparative normal-phase HPLC system the retention times were 6.9 min for *cis*-vitamin K_1, 7.8 min for *trans*-vitamin K_1 and 8.7 min for vitamin K_1 2,3-epoxide. The retention time for the internal standard Cl-K was 7.2 min and it therefore co-eluted with vitamin K_1 (Figure 2.3a). Reversed-phase HPLC produced clear separation of vitamin K_1, vitamin K_1 2,3-epoxide and Cl-K. The

FIGURE 2.3 Determination of vitamin K_1 and vitamin K_1 2.3-epoxide in rat liver.
Normal-phase (semi-preparative) step
(a) Cl-K (100 ng)
(b) extract of 33% liver homogenate (100 µl).
The arrows indicate the period throughout which eluent was collected for subsequent reversed-phase chromatography.
Peak : 1 = Cl-K



retention times were 14.8, 10.4 and 13.8 min respectively (Figure 2.4a). Figure 2.4b illustrates the trace obtained from reversed-phase HPLC analysis of the fraction collected from the normal-phase HPLC system. Hepatic vitamin K_1 and vitamin K_1 2,3-epoxide concentrations were determined by the ratio of vitamin K_1 and vitamin K_1 2,3-epoxide peak heights to the peak height of internal standard. The mean (n = 4) linear regression lines obtained for the standard curves were $y = 1.112\ x + 0.030$, $r = 0.999$ for vitamin K_1 and $y = 0.658\ x + 0.021$, $r = 0.999$ for vitamin K_1 2,3-epoxide.

Intra-assay variation was calculated by the repeated (n = 6) analysis of a single liver homogenate sample containing 20 $\mu g\ g^{-1}$ of vitamin K_1 and vitamin K_1 2,3-epoxide and gave a coefficient of variation of 1.6%. Inter-assay variation was calculated by repeated (n = 6) analysis of a single liver homogenate sample containing 20 $\mu g\ g^{-1}$ of vitamin K_1 and vitamin K_1 2,3-epoxide on separate days over a period of several weeks and gave a coefficient of variation of 6.9%

It is known that *cis*-vitamin K_1 has far less biological activity than *trans*-vitamin K_1 in stimulating clotting factor synthesis (Knauer et al., 1975) and therefore it is of interest to determine the relative amounts of these isomers in the liver. For the measurement of *cis*- and *trans*-vitamin K_1 it would have been advantageous to use the reversed-phase system for the semi-preparative step and normal-phase HPLC for analytical purposes since it has the ability to separate the isomers. However, loss of vitamin K_1 and the epoxide was experienced when a relatively large volume (8 ml) of reversed-phase eluent was evaporated, but not during evaporation of a similar volume of normal-phase eluent. Therefore to determine the *cis*:*trans*

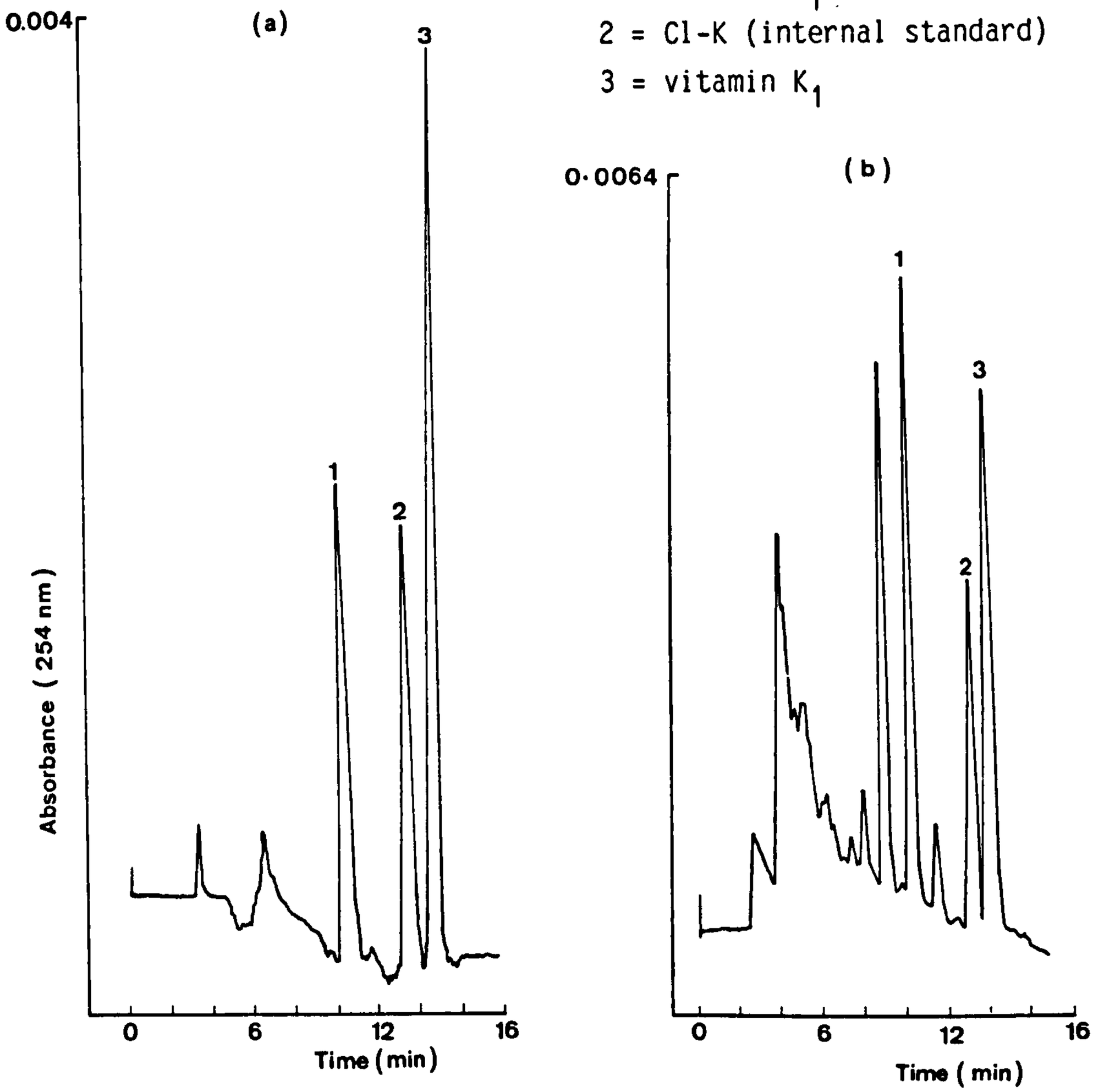
FIGURE 2.4 Determination of vitamin K_1 and vitamin K_1 2.3-epoxide in rat liver.

Reversed-phase (analytical) step.

(a) Separation of 100 ng of vitamin K_1, vitamin K_1 2,3-epoxide and Cl-K

(b) extract of 33% rat liver homogenate (100 µl) obtained 3 h after intravenous administration of vitamin K_1 (1 mg kg^{-1}) and 9 h after intraperitoneal administration of warfarin (63 mg kg^{-1}).

Peaks : 1 = vitamin K_1 2,3-epoxide
2 = Cl-K (internal standard)
3 = vitamin K_1



ratio, a third chromatographic step (normal-phase) was employed to resolve the vitamin K_1 peak (2ml) obtained from the analytical (reversed-phase) system.

2.3.5 Determination of Vitamin K_1 in Plasma by High Performance Liquid Chromatography with Electrochemical Detection

The results of both *in vivo* and *in vitro* studies in which vitamin K_1 and its antagonism by 4-hydroxycoumarin anticoagulants is investigated, have advocated the need to quantify physiological concentrations of the vitamin in order to further our understanding of the pharmacology of vitamin K_1.

The immediate forerunners of methods employed to determine physiological vitamin K_1 concentrations, were multistage assays with UV detection. These were used to determine the vitamin K_1 content of vegetables (Shearer *et al.*, 1980), milk and commercial milk preparations (Haroon *et al.*, 1982). As these assays offered only limited sensitivity (1 to 2 ng) relatively large quantities of starting material had to be extracted and they were not sensitive enough to determine physiological vitamin K_1 concentrations in human plasma. A 2 to 4 fold increase in sensitivity was achieved by Lefevere *et al.* (1979) using a similar multistage assay to that of Shearer and co-workers (1980). This enabled physiological vitamin K_1 concentrations to be determined in a small number of healthy volunteers. Further elaboration of this method resulted in the determination of physiological concentrations of vitamin K_1 in a much larger group of individuals (Lefevere *et al.*, 1982). In the same year, Shearer *et al.* adapted their previous method (Shearer *et al.*, 1980) to determine the concentration of vitamin K_1 in the plasma of healthy adults, healthy mothers at full term and their newborn infants.

At this stage a discrepancy in physiological vitamin K_1 concentrations as determined by different methods began to appear. A 10-fold difference was reported between Shearer *et al.* (1980; 0.10 to 0.66 ng ml^{-1})

and Lefevere et al. (1982; 0.9 to 7.8 ng ml^{-1}) At the present time there is approximately a 100-fold difference in the concentrations of vitamin K_1 detected in healthy volunteers using multistage HPLC with UV detection (Pietersma de Bruyn et al., 1985; 1.35 to 6.75 ng ml^{-1}; Sann et al., 1985; 1 to 140 ng ml^{-1}).

The above results suggest that the methods employed are not specific enough to determine accurately physiological vitmain K_1 plasma concentrations. The search for more selective and more sensitive detection methods resulted in the development of a number of different assays. Several groups have shown greater sensitivity using fluorimetric detection rather than UV detection (Langenberg and Tjaden, 1984; Lambert et al., 1986). The fact that both vitamin K_1 and vitamin K_1 2,3-epoxide can be measured at very low concentrations (120 pg ml^{-1}) provides an additional advantage for this type of assay.

Electrochemical detection in combination with HPLC has been shown to be a powerful approach for the trace measurement of electroactive compounds such as vitamin K_1. Initially the vitamin was detected using a single thin-layer cell containing a glassy-carbon working electrode which was in operation in the reductive mode and thus vitamin K_1 was detected in the form of the quinol (Hart et al., 1984). This method was shown to be three times more sensitive than that developed by Shearer and co-workers using HPLC with UV detection. However it was soon discovered that the use of dual electrode cells further improved the sensitivity (50 pg) and selectivity of the vitamin K_1 assay (Haroon et al., 1984; Hart et al., 1985). This was very important since Hart and co-workers were aiming to detect subnormal plasma concentrations of vitamin K_1 in patients.

The method employed in this thesis to determine concentrations

of vitamin K_1 which were below the limit of sensitivity of the method described in section 2.3.3 was the same as that used by Hart and co-workers (1985) but with slight modification.

Method

Standard solutions of vitamin K_1 were prepared in hexane at concentrations of 5, 50 and 500 μg ml^{-1} for normal-phase HPLC. For the same system a standard solution of the internal standard MK6 was prepared in hexane at a concentration of 1.093 μg ml^{-1}. Standard solutions of vitamin K_1 were prepared in absolute ethanol at concentrations of 0.005, 0.05, 0.5, 5, 50 and 500 μg ml^{-1} for reversed-phase HPLC. For the same system standard solutions of MK6 were prepared in absolute ethanol at concentrations of 3.28, 32.8 and 328 ng ml^{-1}. All standard solutions were stored at room temperature.

An aliquot of internal standard (40 to 600 μl) was added to a glass tube prior to the addition of plasma (0.5 to 1.0 ml). The contents of the tube were vortexed (30s) and left at room temperature to equilibrate (15 min). Absolute ethanol (5 ml) was then added to precipitate plasma proteins immediately followed by the addition of hexane (5 ml). The extraction was carried out by gentle mechanical tumbling (30 r.p.m.; 20 min). To ensure complete separation of the hexane phase from the ethanol-water phase the tube was centrifuged 2000 x g; 2 min). The hexane layer was then carefully removed, transferred to a clean tube and evaporated to dryness under vacuum at 50°C. The extract residue was redissolved in hexane (1.5 ml) and loaded onto a Sep-Pak Silica Cartridge using a glass syringe. The syringe was then washed with further hexane (2 x 10 ml) and the eluate discarded. The fraction containing vitamin K_1 was eluted from the

cartridge with 3% diethyl ether in hexane (10 ml), collected into a clean tube and evaporated to dryness under vacuum at 50°C. The residue extract was then redissolved in normal-phase mobile phase (60 µl). Following the injection of this solution (50 µl) onto the normal-phase HPLC system, the effluent was collected between 6 and 12 min to ensure complete recovery of vitamin K_1 and MK6 (Figure 2.5b). The effluent was evaporated under vacuum at 50°C and then the residue extract was redissolved in methanol (200 µl). An aliquot (100 µl) of this was injected onto the reversed-phase HPLC system for analysis (Figure 2.6b).

The normal-phase semi-preparative column was packed with Partisil 10 ODS (25 cm x 4.5 mm i.d.) and protected by a guard column (2.5 cm x 4.5 mm i.d.) packed with Partisil 10 silica gel. The mobile phase used was 0.34% acetonitrile in hexane at a flow rate of 2 ml min^{-1}. Vitamin K_1 and MK6 were detected by UV absorbance at 254 nm with a sensitivity of up to 0.002 a.u.f.s.

The reversed-phase analytical column was packed with Hypersil C_8 (25 cm x 4.5 mm i.d.). The mobile phase used was 97% methanol with 3% 0.05M acetate buffer (pH 3.0). The addition of 0.05M sodium EDTA (20 ml) enhanced the sensitivity of the system. The flow rate of the mobile phase was 1 ml min^{-1} and this was left recycling overnight with the detector set at the operating potentials but with the cell switches in the "dummy" position.

Detection of vitamin K_1 and MK6 was carried out in the redox mode. The first electrode (upstream electrode) detector 1 was set at -1.30v and at this potential vitamin K_1 was reduced from the quinone form to the hydroquinone form. This reduced form of vitamin K_1 was then detected at the second electrode (downstream electrode), detector

FIGURE 2.5 Determination of vitamin K_1 in human plasma by electrochemical detection. Normal-phase (semi-preparative) step.
(a) separation of 250 ng of vitamin K_1 and MK6.
(b) extract of human plasma (0.5 ml).
The arrows indicate the period throughout which eluent was collected for subsequent reversed-phase chromatography.

Peaks: 1 = *cis* - vitamin K_1
2 = *trans* - vitamin K_1
3 = MK6 (internal standard)

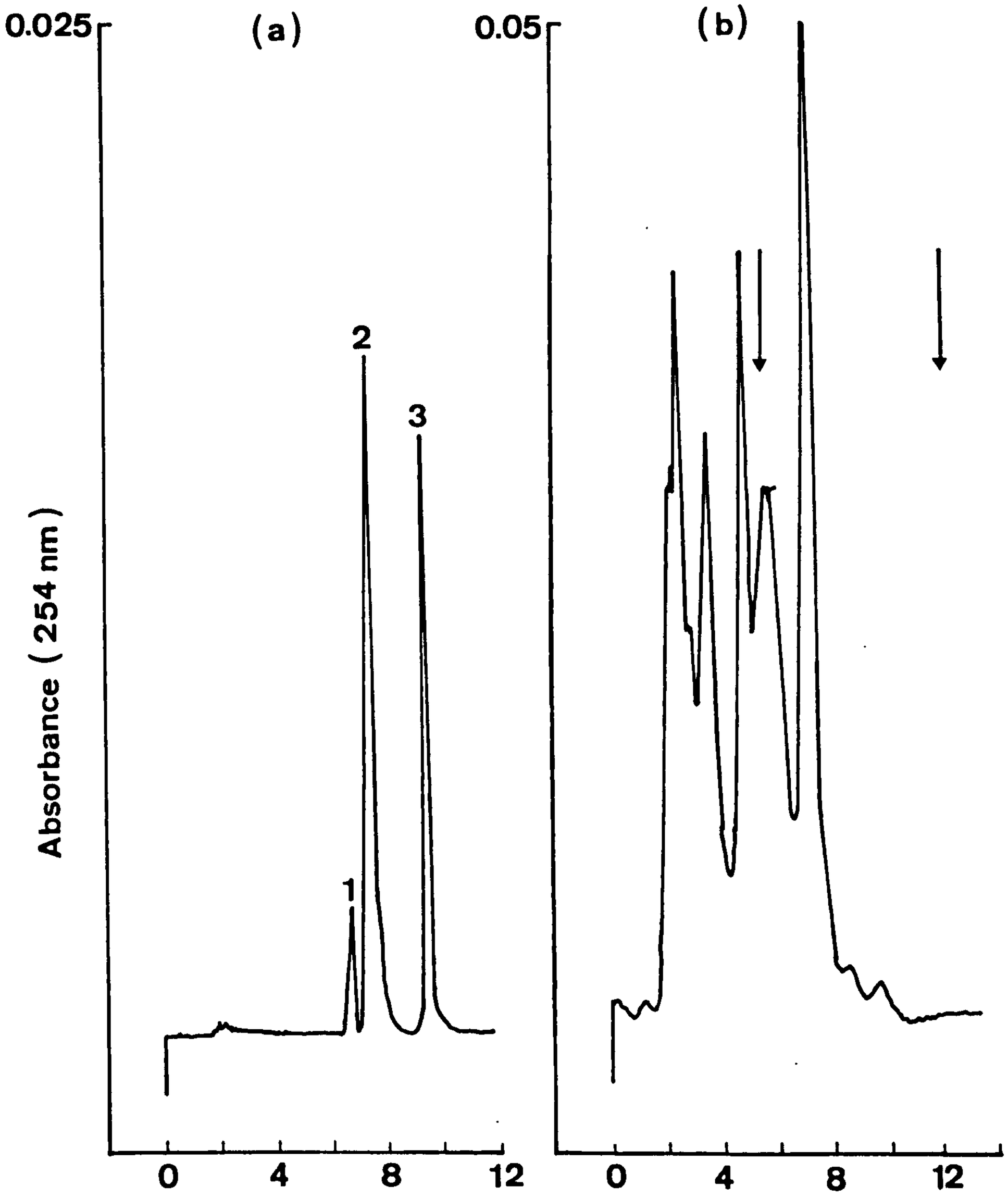
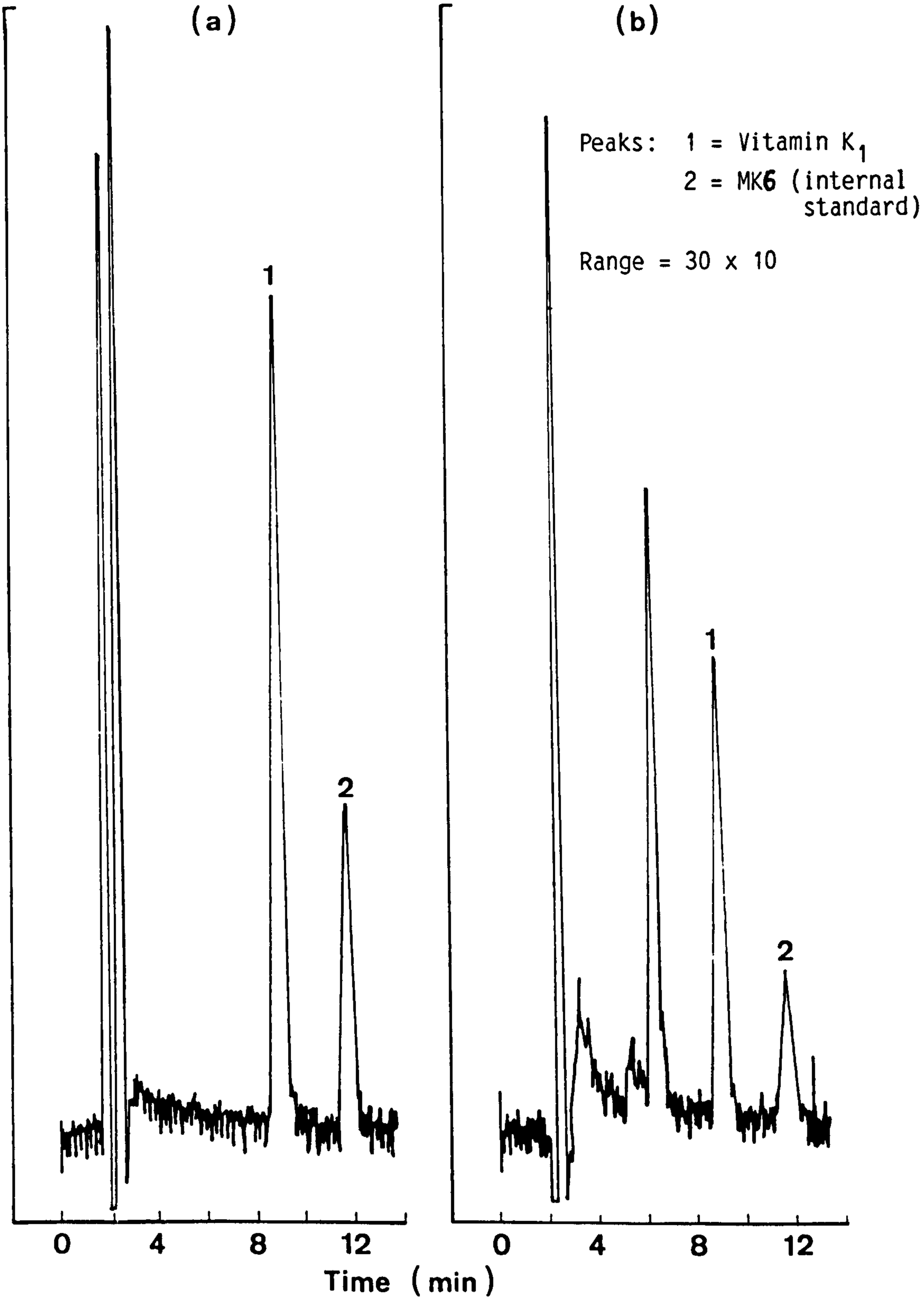


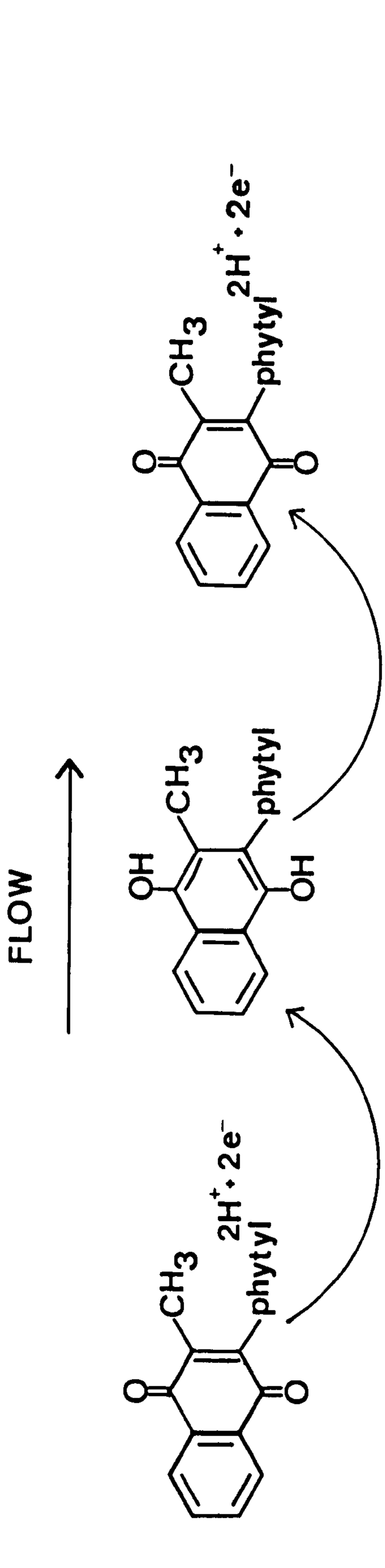
FIGURE 2.6 Determination of vitamin K_1 in human plasma by electrochemical detection. Reversed-phase (analytical) step.
(a) separation of vitamin K_1 (5 ng) and MK6 (3.28 ng)
(b) extract of human plasma (0.5 ml) obtained 13 h after intravenous administration of vitamin K_1 (10 mg kg^{-1}) to an individual on steady state racemic warfarin therapy (1 mg daily).



2, where it was reoxidised to the quinone at a potential of +0.15v (Figure 2.7). To determine the potential which needed to be applied to most effectively detect vitamin K_1, a voltammogram (a current-voltage curve) was constructed by keeping the voltage at detector 1 constant, injecting a fixed volume of standard solution of vitamin K_1 and then altering the voltage at detector 2 until a maximum response i.e. maximum current was obtained. This was then repeated by keeping the voltage at detector 2 constant.

The final overall recoveries for vitamin K_1 and MK6 from plasma were 23% and 21.5% respectively, at a plasma concentration of 25 ng ml^{-1}. The range of concentrations measured using this assay were 0.76 to 2466 ng ml^{-1} with a sensitivity of 0.5 ng ml^{-1}.

Using the semi-preparative normal phase HPLC system, the retention times were 6.7, 7.6 and 9.6 min for *cis*-vitamin K_1, *trans*-vitamin K_1 and MK6 respectively (Figure 2.5a). Reversed-phase HPLC with electrochemical detection produced clear separation of vitamin K_1 and MK6. The retention times were 9 min for vitamin K_1 and 11.8 min for MK6 (Figure 2.6a). Plasma vitamin K_1 was determined by the ratio of vitamin K_1 peak height to the peak height of MK6. The mean ($n = 4$) linear regression lines obtained from the standard curves in human plasma were $y = 1.398x + 0.272$, $r = 0.994$ and in rabbit plasma were $y = 0.919x + 0.327$, $r = 0.999$. Intra-assay variation was calculated by repeated chromatography ($n = 6$) of a rabbit plasma sample and gave a coefficient of variation of 3.4% Inter-assay variation was calculated by repeated chromatography ($n = 6$) of a rabbit plasma sample on separate days over a period of several weeks and gave a coefficient of variation of 8.2%.



Redox reaction employed in the electrochemical detection of vitamin K_1.

FIGURE 2.7

2.3.6 Determination of Vitamin K_1 in Liver By High Performance Liquid Chromatography with Electrochemical Detection

Although the method described in section 2.3.4 was ideal for the determination of hepatic concentrations of vitamin K_1 and vitamin K_1 2,3-epoxide following a pharmacological dose of the vitamin, the limit of sensitivity of this method is much too high (250 ng g^{-1}) to be used for the determination of physiological concentrations of the vitamin in the liver.

There are few reports in the literature in which physiological hepatic concentrations of vitamin K_1 have been determined. Initially multi-dimensional classical chromatographic techniques such as adsorption, reversed-phase (Matschiner et al., 1967) and argentation chromatography (Matschiner and Amelotti, 1968) were used. These methods were not always reliable due to the limitations of sensitivity and the difficulty in obtaining pure fractions of K vitamins for quantification by UV spectroscopy.

More recently, Haroon and Hauschka (1983) described the application of an HPLC method with UV detection for the determination of physiological vitamin K_1 concentrations in rats. By extracting 7 to 10 g of liver they determined endogenous vitamin K_1 in male rat liver to be 8 ng g^{-1}. This method was rapidly followed by an assay which used the same purification method, but which employed electrochemical instead of UV detection (Haroon et al., 1984). This method offered a limit of sensitivity of approximately 100 pg. Furthermore, the system also offered superior selectivity over UV detection.

Consequently, the method employed in this thesis to determine endogenous concentrations of vitamin K_1 in rabbit liver utilized HPLC in conjunction with electrochemical detection.

Method

The standard solutions of vitamin K_1 and the internal standard, MK6 were the same as those described in Section 2.3.5.

To an aliquot of liver homogenate (1.5 ml), internal standard solution was added (65.6 ng). The suspension was vortexed (30 s) and left at room temperature to equilibrate (15 min). Acetone (2 ml) was then added and the mixture was mechanically shaken (20 min). Hexane (5 ml) was added, the contents of the tube shaken (10 min) and then centrifuged (5000 x g; 5 min) to ensure complete separation of the organic layer from the aqueous layer. The organic layer was removed, evaporated to dryness under vacuum at 50°C and then the extract residue was redissolved in normal phase eluent (60 µl). Analysis of the liver extract was then performed as described in Section 2.3.5.

The final overall recoveries of vitamin K_1 and MK6 from rabbit liver homogenate were 26% and 22% respectively, at a liver concentration of 1.0 g ml^{-1}. The range of concentrations determined using this assay were 26.8 to 181.4 ng g^{-1} with a limit of sensitivity of 0.5 ng g^{-1}. The mean (n = 3) linear regression line obtained from the standard curve in rabbit liver homogenate was $y = 1.392x + 0.404$, $r = 0.998$. Intra-assay variation was calculated by repeated chromatography (n = 6) of a rabbit liver homogenate sample and gave a coefficient of variation of 6.4%. Inter-assay variation was calculated by repeated chromatography (n = 4) of a rabbit liver homogenate sample on separate days over several weeks and gave a coefficient of variation of 7.9%.

2.3.7 Determination of Prothrombin Time for Rat, Rabbit and Human Plasma.

Method

Prothrombin time was determined by the method of Quick (1957). Blood samples (0.54 ml from rat and 0.9 ml from rabbit and man) were collected into 3.8% w/v trisodium citrate solution (0.06 or 0.1 ml) in polypropylene tubes and immediately centrifuged (8000 x g; 2 min). Thromboplastin (0.1 ml) was added to citrated plasma (0.1 ml) and incubated at 37°C for 2 min, in duplicate. Calcium chloride (0.025M; 0.1 ml) was added and the clotting time determined using a Schnitger and Gross coagulometer (Burkard Scientific Ltd., Uxbridge).

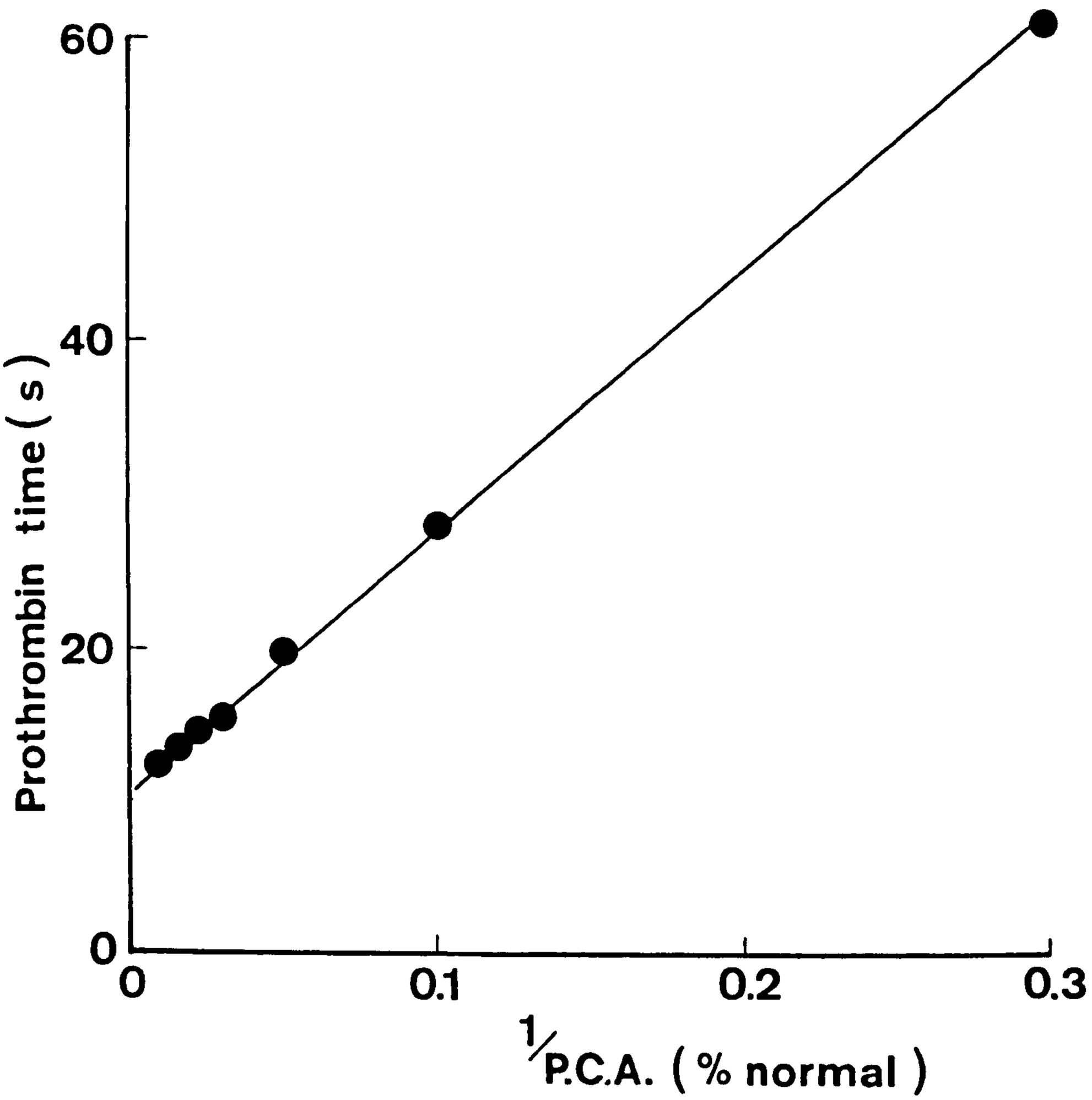
Standard curves for prothrombin complex activity (P.C.A.) in rat (Figure 2.8) and rabbit plasma were obtained by determining the clotting times of pooled normal citrated plasma, diluted with adsorbed plasma, at concentrations of 1 to 100%. Adsorbed plasma was prepared by repeatedly mixing pooled normal citrated plasma with aluminium hydroxide followed by centrifugation (8000 x g; 10 min), until the prothrombin time of the adsorbed plasma exceeded 500 s. This indicated that the vitamin K-dependent clotting factors II, VII, and X had been removed. P.C.A. for each animal was expressed as a percentage of its own control, taking the value obtained at the beginning of each experiment as 100%. The coefficient of variation for the determination of prothrombin time by this method in rat, rabbit and human plasma were 1.6, 1.2 and 1.2% respectively. The mean (n = 4) linear regression line obtained from the standard curve for rabbit plasma was $y = 250x + 6.3$, $r = 0.989$.

FIGURE 2.8 Standard curve of prothrombin time (PT) vs $^{1}/$prothrombin complex activity (P.C.A.) for the determination of P.C.A. in rat plasma.

Each point represents the mean of four determinations. After linear regression analysis

$$r = 0.999, \quad m = 203.4, \quad c = 9.8$$

$$\text{thus P.C.A. (\%)} = \frac{203.4}{PT - 9.8}$$



2.3.8 Determination of the Effect of Cimetidine on the Plasma Protein Binding of Warfarin In Vitro.

Method

Equilibrium dialysis was performed using the Dianorm equilibrium system (Weder and Bickel,1971). This comprises of a semi-permeable membrane which separates a Teflon dialysis cell into two halves.

Dialysis membranes were cut to size from Visking cellophane tubing. The membranes were soaked in distilled water overnight and subsequently soaked in 30% ethanol for 30 min, washed thoroughly in distilled water and finally soaked in isotonic sodium phosphate-chloride buffer (ph 7.4, 0.1M) for 30 min. Dialysis cells were prepared by placing a membrane, gently dried with tissue, between the two halves of the Teflon dialysis cell, which was then fixed to the cell carrier.

Equal volumes (0.9 ml) of blank human plasma and phosphate buffer containing non-radiolabelled warfarin (500 ng ml^{-1}), [^{14}C]-warfarin (< 0.1 µCi, specific activity 55 mCi $mmol^{-1}$) and cimetidine at concentrations ranging from 0.1 to 100 µg ml^{-1} were injected into the two sides of the dialysis cell. Dialysis was performed for 16 h at 37°C. At the end of this time, the cells were emptied and the radioactivity of equal aliquots (0.5 ml) of plasma and buffer were determined by liquid scintillation counting.

The concentration of free warfarin in plasma samples was determined using the method described by Giacomini et al. (1984) which takes into account the effect of volume shift during dialysis.

$$\text{Fraction unbound} = \frac{C'_B}{C^o_{bnd} + C'_B}$$

Where C'_B is the concentration of free warfarin in the buffer after dialysis and C^o_{bnd} is the concentration of bound drug had no volume shift occurred. The value of C^o_{bnd} is obtained from

$$C^o_{bnd} = C'_{bnd} (1 + \delta)$$

Where C'_{bnd} represents the bound drug concentration obtained after dialysis. δ is the fractional increase in the volume of the plasma due to the osmotic water shift and can be approximated as follows:

$$\delta = \frac{P}{P'} - 1$$

Where P and P' are the protein concentrations in the plasma before and after dialysis. In this experiment the value of δ was 0.13.

2.4 PHARMACOKINETIC ANALYSIS OF RESULTS.

Elimination Half-Life

The elimination half-life is the time taken for the plasma concentration, as well as the amount of drug in the body, to fall by one half after attaining distribution equilibrium. This value is constant for a drug obeying first-order elimination kinetics and is independent of the amount of drug in the body. Therefore, less drug is eliminated in each succeeding half-life, and theoretically the drug can never be completely eliminated. However, in practice, all the drug (97%) may be regarded as having been eliminated by 5 half-lives.

The elimination half-life may be determined from the semi-logarithmic plot of drug concentration versus time. The elimination phase is represented by the linear equation.

$$\log Cp = \log Cp_o - K_s.t \qquad (1)$$

where Cp is the drug concentration at time t, Cp_o is the drug concentration at time O and K_s is the slope of the line.

If equation 1 is converted to natural logarithms this produces:

$$Ln\ Cp = Ln\ Cp_o - K.t \qquad (2)$$

where K is the elimination rate constant equal to K_s x 2.303. The half-life is the time required for the drug concentration to fall by one-half, thus if $Cp = \frac{Cp_o}{2}$ and $t = t_{\frac{1}{2}}$, substitution into equation 2 produces:

$$K.t_{\frac{1}{2}} = Ln\ 2 = 0.693 \text{ or } t_{\frac{1}{2}} = 0.693/K \qquad (3)$$

Apparent Volume of Distribution

Apparent volume of distribution is the apparent volume into which a drug distributes in the body at equilibrium. The extent of distribution can be determined by relating the plasma concentration to a known amount of drug in the body. Thus, the apparent volume of distribution, V_d, is the volume of plasma at the drug concentration, Cp, required to account for all the drug in the body, Ab

$$V_d = {Ab}/{Cp} \qquad (4)$$

Apparent volume of distribution rarely corresponds to a real physiological volume and is largely dependent on the plasma and tissue binding of the drug. In fact, the binding to tissue components may be so great that the volume of distribution is many times the total body size.

Clearance

Clearance is the volume of a biological fluid from which drug is removed per unit time. The clearance of a drug relates the concentration of that drug to its rate of elimination. The value of clearance is dependent upon the site of measurement of concentration but is independent of concentration for drugs which obey first-order elimination kinetics.

At equilibrium

$$\text{Rate of elimination} = Clp \times Cp \qquad (5)$$

Where Clp is plasma clearance and Cp is plasma concentration.

In the following chapters of this thesis, after a single

intravenous, or oral dose of either racemic, R(+) or S(-) warfarin to man or the rabbit, the area under the plasma concentration-time curve, up to the last observation, was determined by the trapezoidal rule and the terminal area until infinity by extrapolation, by dividing the last observation by the terminal elimination rate constant. The area under the plasma concentration-time curve for vitamin K_1 2,3-epoxide following a pharmacological dose of vitamin K_1 in the presence of warfarin was also determined by this method.

The pharmacokinetic parameters of warfarin were calculated assuming a one compartment model. The apparent volume of distribution was calculated as follows:

$$V_d = \text{dose} / \text{AUC} \times K \qquad (6)$$

Where AUC is the area under the plasma concentration-time curve to infinity and K is the elimination rate constant.

Equation 5 illustrated the relationship between plasma clearance and rate of elimination.

Furthermore

$$\text{Rate of elimination} = K \times Ab \qquad (7)$$

Where K is the elimination rate constant and Ab is the amount of drug in the body (from equation 4, $Cp \times V_d$). Therefore, combining equations 4, 5 and 7:

$$Clp = V_d \times K \qquad (8)$$

which is the equation used to determine plasma clearance of warfarin in this thesis.

After a single intravenous dose of vitamin K_1 to rabbits, a bi-exponential equation was fitted to the vitamin K_1 plasma concentration-time data (excluding the observation at t = 0.5 h) using a regression analysis programme (Nielsen-Kudsk, 1980) and from the two first order rate constant determined, the β and γ plasma half-lives were calculated using equation 3.

CHAPTER 3

A STUDY OF THE RELATIONSHIP BETWEEN THE PHARMACOKINETICS AND THE PHARMACODYNAMICS OF RACEMIC WARFARIN AND ITS ENANTIOMERS IN THE RABBIT

3.1 Introduction

One of the major problems associated with warfarin therapy is the inter-individual variation in pharmacological response to a standard dose (Breckenridge, 1977). This variation is largely due to differences in the pharmacokinetics (O'Reilly et al., 1963; O'Reilly and Aggeler, 1968) but can also be due to differences in the pharmacodynamic response to this compound (Alving et al., 1984).

Clinically warfarin is administered as a racemic mixture of the enantiomers R(+) and S(-) warfarin. Previous studies in man have concluded that the administration of racemic warfarin is effectively like giving two separate drugs, due to the differing pharmacokinetic and pharmacodynamic properties of R(+) and S(-) warfarin (Hewick and McEwen, 1973; Breckenridge et al., 1974).

The proposed site of action of warfarin is the enzyme, vitamin K epoxide reductase (Whitlon et al., 1978). This enzyme is responsible for the regeneration of vitamin K_1 from vitamin K_1 2,3-epoxide in the vitamin K epoxide cycle (Bell and Matschiner, 1972). Vitamin K acts as a co-factor in the post-ribosomal synthesis of clotting factors II, VII, IX and X (Jackson and Suttie, 1977).

For many drugs including tubocurarine and succinylcholine, pharmacological effect at any time can be related to plasma concentration (Levy, 1964; Levy, 1967). This, however is not the case for warfarin due to its effect being indirect; that is warfarin inhibits vitamin K epoxide reductase which subsequently decreases clotting factor synthesis. Thus, there is no direct relationship between the pharmacological response, as determined by P.C.A. and the plasma concentration of warfarin (Nagashima et al., 1969).

The aim of the study described in this chapter was to develop the rabbit as an animal model which could be used to evaluate the relative contributions of the pharmacokinetics and the pharmacodynamics of any 4-hydroxycoumarin anticoagulant to the pharmacological effect of that drug observed *in vivo*. In the present study this model was used to investigate the pharmacology of warfarin and its enantiomers.

3.2 Development of Animal Model

Previous work has demonstrated the rabbit to be a useful animal model for the determination of the pharmacodynamics of warfarin and the pharmacological effect of vitamin K_1 in coumarin anticoagulated animals over a period of several days (Park et al., 1980; Park and Leck, 1982).

As a consequence of the ability to take sequential blood samples over a prolonged period of time, the rabbit was chosen as the animal in which the pharmacokinetics and the pharmacodynamics of warfarin and its enantiomers were determined simultaneously and in the same animal.

Blood samples were taken by making a small incision into the right marginal ear vein, thus the animals remained conscious throughout the entire experiment. Blood flow from the incision could be easily halted by the application of gentle pressure and started again by gently wiping the original cut.

3.3 Methods

The Pharmacokinetic Study

Groups of rabbits received single intravenous doses of either racemic warfarin (0.63 mg kg^{-1}; n = 4 or 6.3 mg kg^{-1}; n = 8), R(+) warfarin (0.63 mg kg^{-1}; n = 4) or S(-) warfarin (0.63 mg kg^{-1}; n = 4) in 0.9% w/v NaCl solution (0.5 ml kg^{-1}) via the left marginal ear vein. Blood samples (3 ml) were collected into heparinised blood tubes from the right marginal ear vein at 0, 4, 8, 12, 16, 20, 24, 28, 32, 38, 42, 46, 50, 54, 60, 72 and 168 h following racemic warfarin (6.3 mg kg^{-1}) and at 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 48, 52, 60, 72 and 168 h following racemic, R(+) and S(-) warfarin (0.63 mg kg^{-1}). Plasma was obtained by centrifugation (2000 x g; 20 min) and stored frozen (-20°C) until required for analysis as described in section 2.3.1.

Pharmacokinetic parameters were calculated from the plasma concentration - time data as described in section 2.4. Results are expressed as mean ± standard error of the mean (mean ± s.e.). Levels of significance were determined using the non-paired Student's t test.

The Pharmacodynamic Study

Blood samples (0.9 ml were collected into 3.8% w/v trisodium citrate solution (0.1 ml), from the right marginal ear vein of the animals described in the pharmacokinetic study, at 0, 4, 8, 12, 16, 20, 24, 28, 32, 38, 46, 50, 54, 60, 70 and 94 h following racemic warfarin (6.3 mg kg^{-1}), at 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 48, 56 and 80 h following racemic warfarin (0.63 mg kg^{-1}) and at 0, 4, 8, 12, 16, 20, 24, 28, 32, 36, 48 and 52 h following R(+) and S(-) warfarin (0.63 mg kg^{-1}) and also at 72 h following R(+) warfarin, for the immediate

determination of P.C.A., as described in section 2.3.7. Results are expressed as mean ± standard error of the mean (mean ± s.e.).

3.4 Results

Pharmacokinetics of Racemic Warfarin and its Enantiomers

The plasma concentration - time curves following intravenous administration of single doses of racemic warfarin (6.3 and 0.63 mg kg^{-1}) can be seen in Figure 3.1. Plasma concentrations fell mono-exponentially in both cases. There was no significant difference in the plasma half-lives obtained for these two doses of racemic warfarin (Table 3.1); indicating that the pharmacokinetics of racemic warfarin are independent of dose in the rabbit, over the ten-fold dose range investigated in this study. The plasma concentration - time curves following intravenous administration of a single dose of R(+), S(-) and racemic warfarin at 0.63 mg kg^{-1}, can be seen in figure 3.2. The plasma concentrations of R(+) warfarin were always higher than those for S(-) warfarin. The plasma concentration - time curve obtained for racemic warfarin (0.63 mg kg^{-1}) fell between that obtained for the enantiomers of warfarin at the same dose.

The pharmacokinetic parameters obtained for the enantiomers of warfarin are shown in Table 3.1. The plasma half-life of S(-) warfarin was significantly ($P < 0.001$) shorter than that of R(+) warfarin and plasma clearance of S(-) warfarin was approximately 4 times greater than that of R(+) warfarin. The apparent volume of distribution of S(-) warfarin was more than twice that of R(+) warfarin.

Pharmacodynamics of Racemic Warfarin and its Enantiomers

In order to investigate the duration of action of racemic warfarin and its enantiomers, preliminary experiments had to be carried out to determine a dose of anticoagulant sufficient to produce maximum

FIGURE 3.1 Plasma concentrations of racemic warfarin vs. time in rabbits following intravenous administration of 6.3 mg kg^{-1} racemic warfarin (● ; n = 8) and 0.63 mg kg^{-1} racemic warfarin (○ ; n = 4).

Results are expressed as means and vertical bars show s.e. mean.

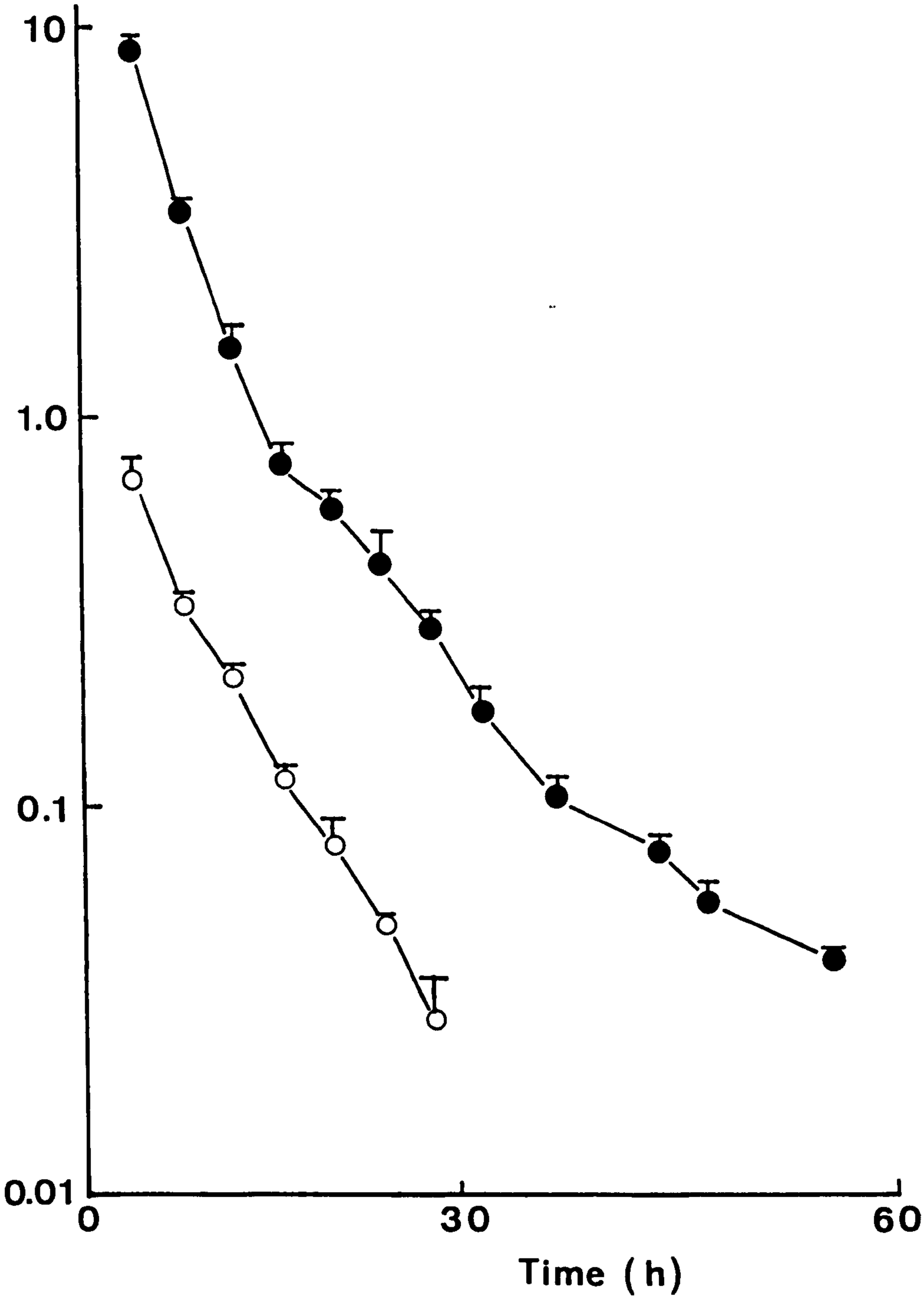


FIGURE 3.2 Plasma concentrations of warfarin vs. time in rabbits following intravenous administration of 0.63 mg kg^{-1} racemic warfarin (○) R(+) warfarin (■) and S(-) warfarin (★).

Results are expressed as means (n = 4) and vertical bars show s.e. mean.

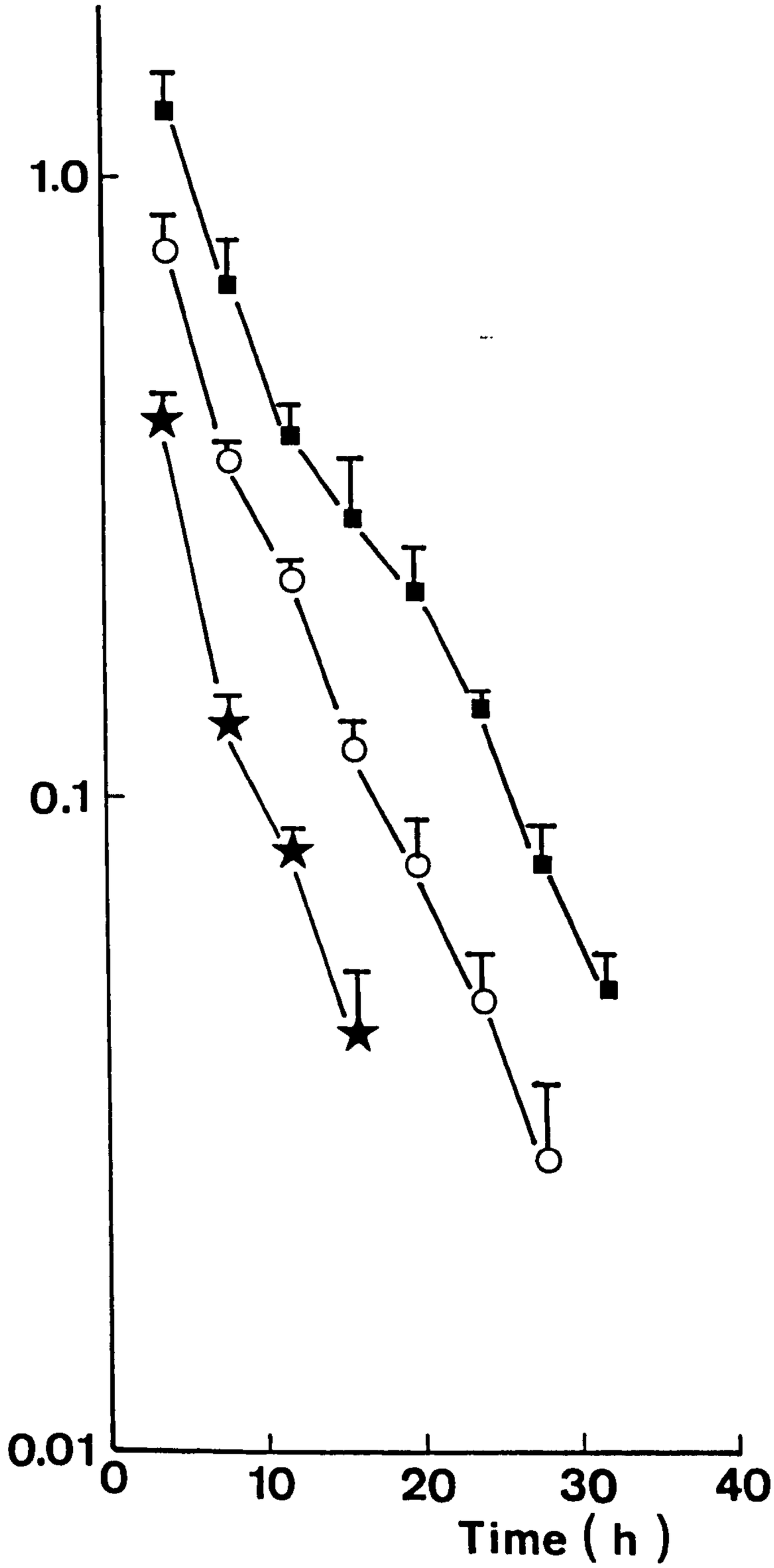


TABLE 3.1. Pharmacokinetic parameters of racemic warfarin and its enantiomers in the rabbit.

	Dose (mg kg^{-1})	$t_{\frac{1}{2}}$ (h)	Cl_p (ml min^{-1} kg^{-1})	V_d (l kg^{-1})
Racemic warfarin n = 8	6.30	5.60 $\pm$ 0.65	1.33 $\pm$ 0.08	0.65 $\pm$ 0.10
Racemic warfarin n = 4	0.63	7.65 $\pm$ 2.19	1.17 $\pm$ 0.12	0.70 $\pm$ 0.12
R(+) Warfarin n = 4	0.63	6.39 $\pm$ 0.18	0.73 $\pm$ 0.04	0.41 $\pm$ 0.03
S(-) Warfarin n = 4	0.63	4.27 $\pm$ 0.28	2.82 $\pm$ 0.27	0.96 $\pm$ 0.09

Values show mean $\pm$ standard error of mean

$t_{\frac{1}{2}}$ - plasma half-life

Cl_p - plasma clearance

V_d - apparent volume of distribution

inhibition of clotting factor synthesis in the rabbit for at least 16 h after administration, but from which recovery would take place without recourse to vitamin K_1. A dose of 0.63 mg kg^{-1} of either racemic, R(+) or S(-) warfarin was found to fulfil this criteria.

The initial half-lives of degradation of P.C.A. after administration of racemic, R(+) and S(-) warfarin were 6.8 $\pm$ 0.3 h, 6.3 $\pm$ 1.3 h and 6.2 $\pm$ 0.8 h respectively (Table 3.2). These values correspond to the maximum rate of degradation of P.C.A., and thus indicate complete inhibition of clotting factor synthesis (Park et al., 1979; Park et al., 1980). The ten-fold greater dose of racemic warfarin (6.3 mg kg^{-1}) produced an initial half-life of degradation of 6.6 $\pm$ 0.5 h, indicating that 0.63 mg kg^{-1} is sufficient to produce maximum inhibition of clotting factor synthesis. Figure 3.3 illustrates that P.C.A. begins to recover 16 h following administration of S(-) warfarin, whereas recovery only begins to take place 28 h after administration of the same dose of R(+) warfarin (0.63 mg kg^{-1}). The recovery of P.C.A. begins 24 h after administration of racemic warfarin (0.63 mg kg^{-1}), however after a ten-fold greater dose of racemic warfarin, it takes a further 8 h for P.C.A. to begin to return to normal (Figure 3.4). These results suggest that S(-) warfarin has a shorter duration of action than R(+) warfarin in the rabbit.

There is no direct relationship between P.C.A. and the plasma concentration of warfarin, because the net rate of change of P.C.A. (R_{net}) is dependent on both the rate of P.C.A. synthesis (R_{syn}), and the rate of P.C.A. degradation (R_{deg}) according to equation 1 (Nagashima et al., 1969).

$$R_{net} = R_{syn} - R_{deg} \qquad (1)$$

$$R_{deg} = K_d.P \qquad (2)$$

TABLE 3.2 Half-lives of degradation of prothrombin complex activity in the rabbit after single intravenous doses of racemic warfarin and its enantiomers.

	Dose ($mg\ kg^{-1}$)	$t_{\frac{1}{2}}$ degradation P.C.A. (h)
Racemic warfarin	6.30	6.6 ± 0.5
Racemic warfarin	0.63	6.8 ± 0.3
R(+) Warfarin	0.63	6.3 ± 1.3
S(-) Warfarin	0.63	6.2 ± 0.8

Values show mean $\pm$ standard error of mean (n = 4)

FIGURE 3.3 Prothrombin complex activity (P.C.A.) vs. time in rabbits following single intravenous doses of 0.63 mg kg^{-1} R(+) warfarin (■) and 0.63 mg kg^{-1} S(-) warfarin (★).

Results are expressed as means (n = 4) and vertical bars show s.e. mean.

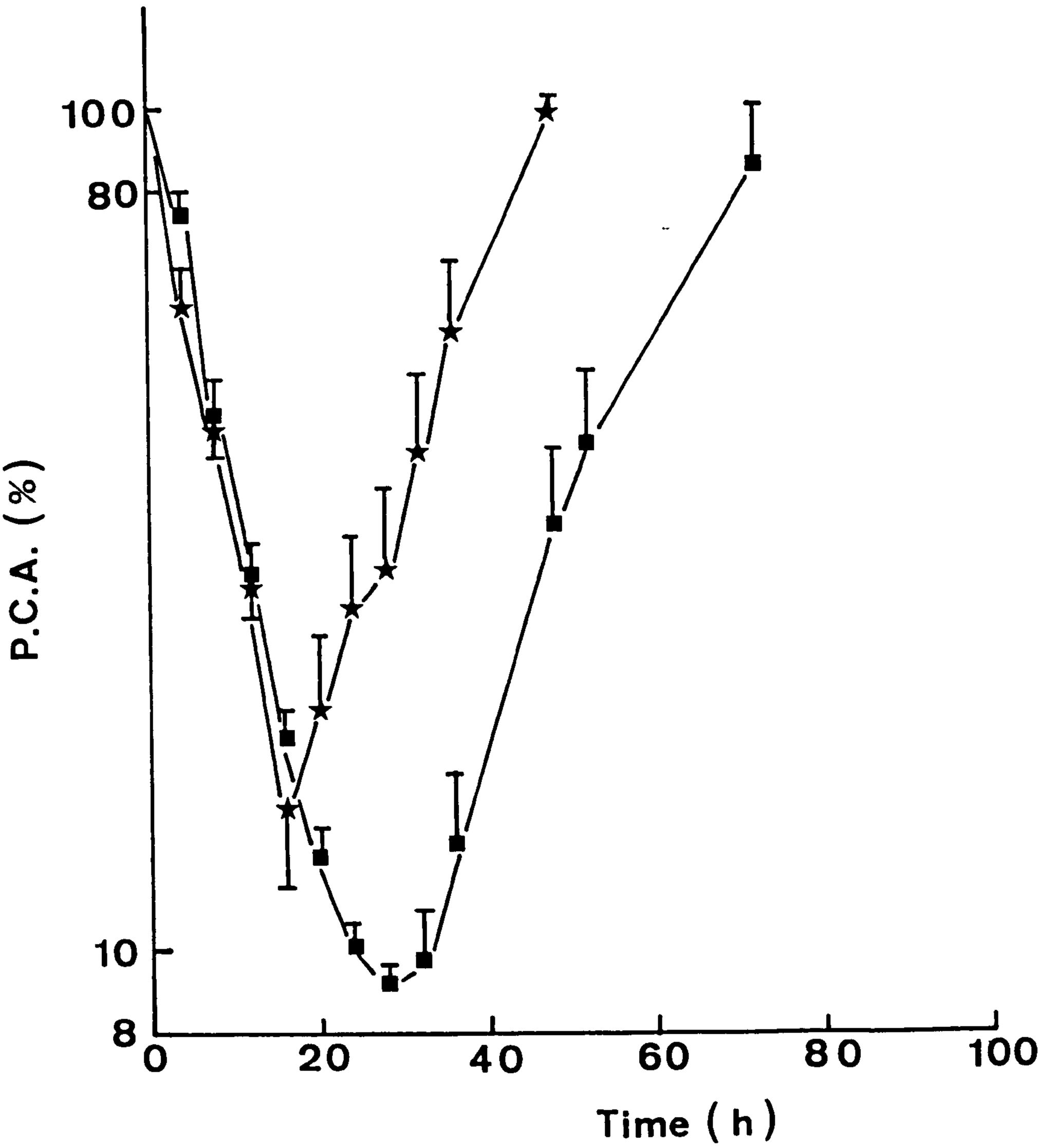
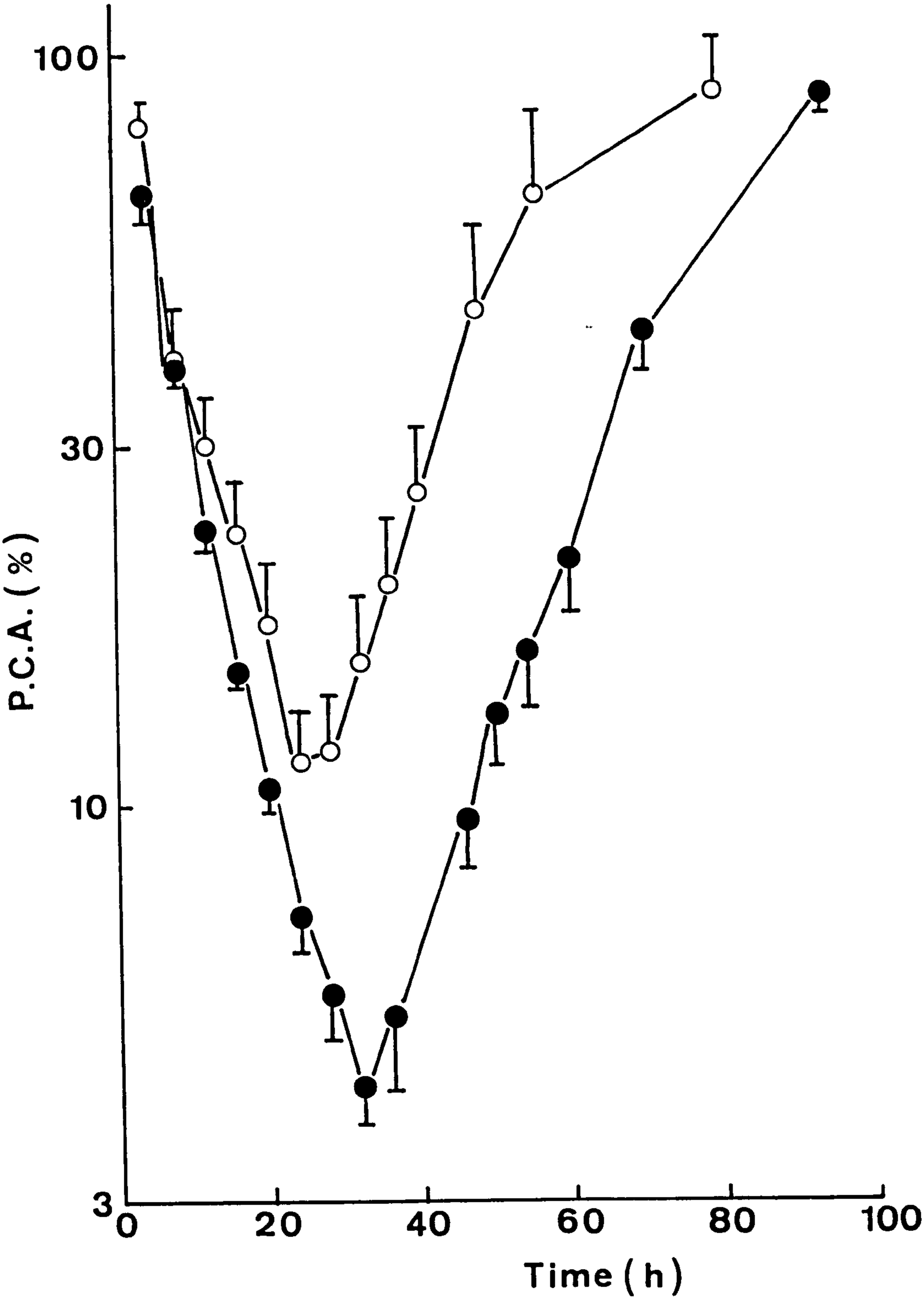


FIGURE 3.4 Prothrombin complex activity (P.C.A.) vs. time in rabbits following single intravenous doses of 6.3 mg kg^{-1} racemic warfarin (●) and 0.63 mg kg^{-1} racemic warfarin (○).

Results are expressed as means (n = 4) and vertical bars show s.e. mean.



Equation 2 (Nagashima et al., 1969) illustrates that R_{deg} can be calculated by multiplying the rate constant of degradation (K_d) by the value of P.C.A. at the time at which R_{deg} is being calculated (P). K_d can be determined, for each animal, when degradation of P.C.A. is maximal, that is during the first 16 h after administration of anticoagulant.

4-hydroxycoumarin anticoagulants only affect clotting factor synthesis not clotting factor degradation (Aggeler and O'Reilly, 1966). Thus R_{syn} is a better pharmacological end point than P.C.A. for the determination of the pharmacological effect of warfarin.

Using equation 3 (Nagashima et al., 1969), R_{syn} may be calculated at various times following warfarin administration.

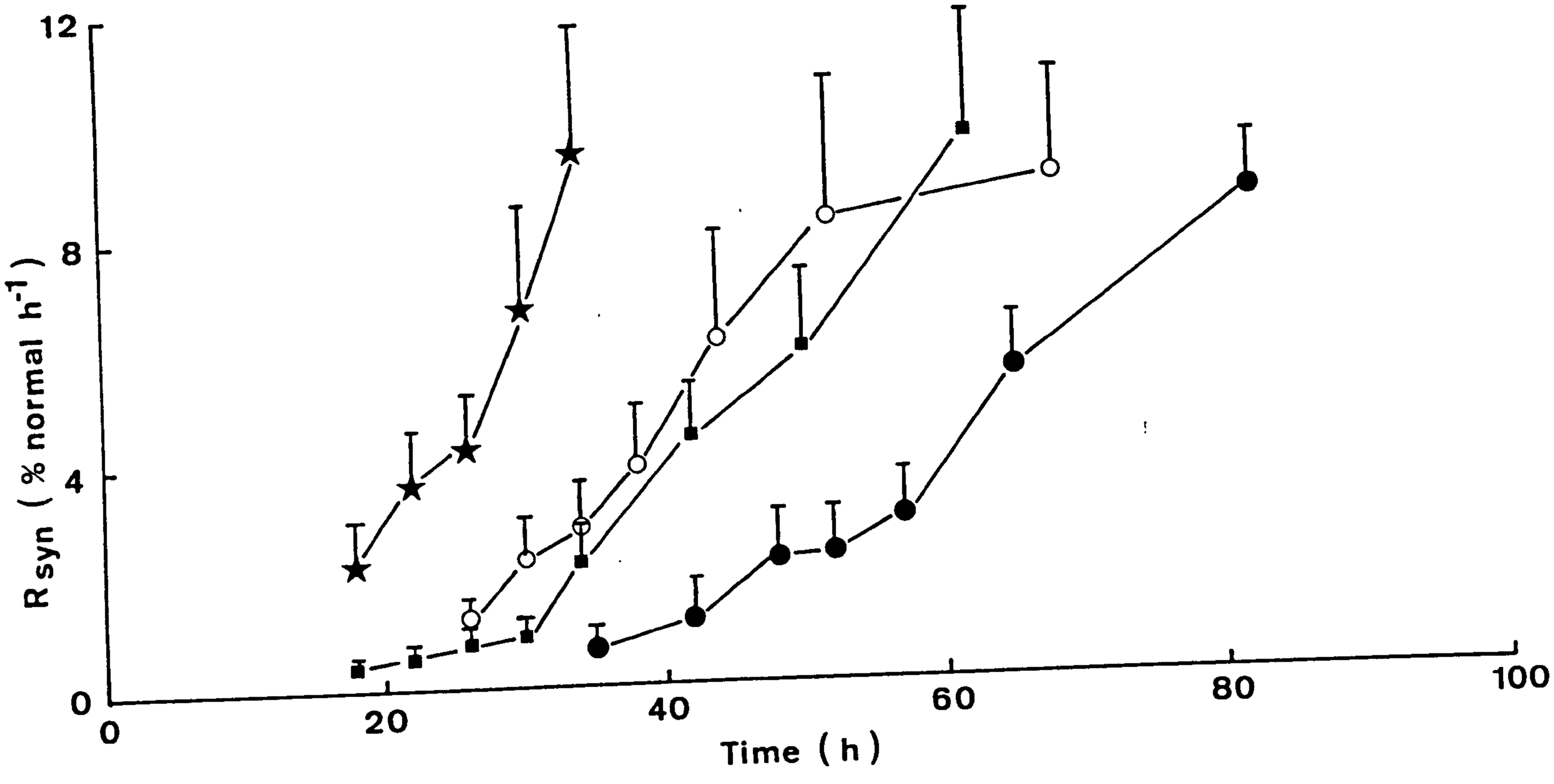
$$R_{syn} = R_{net} + K_d.P \qquad (3)$$

Figure 3.5 illustrates that R_{syn} recovers mono-exponentially following racemic (0.63 and 6.3 mg kg^{-1}), R(+) and S(-) warfarin (0.63 mg kg^{-1}). The recovery of R_{syn} following S(-) warfarin was much more rapid than that following R(+) or racemic warfarin. Furthermore, R_{syn} begins to recover later following the higher dose of racemic warfarin, however once recovery begins, the rate is the same for both doses. Thus the rate of recovery of R_{syn} is independent of dose over the ten-fold dose range employed in this study.

According to current concepts, the anticoagulant effect of warfarin will subside when the concentration of drug at the receptor, associated with vitamin K epoxide reductase, falls (Bell, 1978; Park et al., 1979). Theoretically, for a drug distributed in a single compartment and eliminated by first order kinetics, the intensity of effect (I) is given by equation 4 (Rowland and Tozer, 1980).

FIGURE 3.5 Rate of synthesis of prothrombin complex activity (R_{syn}) vs. time in rabbits following intravenous administration of 6.3 mg kg^{-1} racemic warfarin (●), 0.63 mg kg^{-1} racemic warfarin (○), 0.63 mg kg^{-1} R(+) warfarin (■) and 0.63 mg kg^{-1} S(-) warfarin (★).

Results are expressed as means (n = 4) and vertical bars show s.e. mean.


12
8
4
0
R_{syn} (% normal h^{-1})
0
20
40
60
80
100
Time (h)

$$I = I_o - \frac{m.k.t}{2.3} \qquad (4)$$

Where m is the slope of the intensity-log (amount of drug in the body) curve, k is the elimination rate constant for the drug, and I_o is the intensity of response when Ab_o is the amount of drug in the body. According to this model the intensity of effect, between 20 and 80% of maximum, falls linearly with time. The rate of decline of intensity (ΔI) depends on both the slope of the intensity-log (amount of drug in the body) curve (m) and the elimination half-life of the drug according to equation 5.

$$\Delta I = \frac{m.k.}{2.3} \qquad (5)$$

After intravenous administration of racemic warfarin and its enantiomers, plasma concentrations fell mono-exponentially (Figures 3.1 and 3.2, Table 3.1), the drug was distributed in a single compartment and the pharmacological effect (depression of R_{syn}; Figure 3.5) fell linearly with time. Thus by applying equation 5 to the data obtained following administration of racemic warfarin and its enantiomers, the calculation of m is effectively the determination of the slope of the concentration-response curve for the anticoagulants in vivo.

The value of m determined for racemic warfarin was found to be the same after 0.63 mg kg^{-1} (48.3 $\pm$ 11.6) as it was following 6.3 mg kg^{-1} (50.3 $\pm$ 9.9). In addition, the values of m obtained for R(+) and S(-) warfarin were very similar to each other and also very similar to those obtained for racemic warfarin (Table 3.3). However it must be stressed that by applying equation 5, the assumption that the drug is cleared from the receptor at the same rate as it is cleared from the plasma is made.

TABLE 3.3 'm', the slope of the concentration - response curves for racemic warfarin, R(+) warfarin and S(-) warfarin in the rabbit.

	Dose ($mg\ kg^{-1}$)	m value
Racemic warfarin	6.30	50.3 ± 9.9
Racemic warfarin	0.63	48.3 ± 11.6
R(+) Warfarin	0.63	63.3 ± 14.0
S(-) Warfarin	0.63	46.5 ± 8.2

Values show mean $\pm$ standard error of mean (n = 4)

Relationship Between the Pharmacokinetics and Pharmacodynamics of Racemic Warfarin and its Enantiomers

By relating the pharmacodynamic data to the pharmacokinetic data, the minimum plasma concentration of racemic, R(+) and S(-) warfarin required to completely inhibit clotting factor synthesis was determined. This parameter is referred to as Cp_{max} and was determined by the graphical method illustrated in Figure 3.6. Graphs were constructed for each individual animal using the plasma concentration data and the rate of synthesis of P.C.A. data. The upper portion of the graph illustrates the linear plot of R_{syn} versus time, and the lower portion of the graph illustrates the semi-logarithmic plot of plasma concentration of anticoagulant versus time. The time at which $R_{syn} = 0$, is the time at which the plasma concentration of warfarin is the minimum required to maximally inhibit clotting factor synthesis.

Table 3.4 illustrates that there was no difference in Cp_{max} values over the ten-fold dose range of racemic warfarin employed in this study. This indicates that the potency of racemic warfarin is independent of dose. The Cp_{max} value obtained for S(-) warfarin was below the limit of sensitivity of the assay (<50 ng ml^{-1}), and it can be stated that S(-) warfarin is at least 4 times more potent than R(+) warfarin in the rabbit. R(+) warfarin appears to have a similar potency to racemic warfarin in this species.

FIGURE 3.6 Graphical determination of the minimum plasma concentration of anticoagulant required to completely inhibit clotting factor synthesis (Cp_{max}). Data for an individual rabbit following 0.63 mg kg^{-1} racemic warfarin.

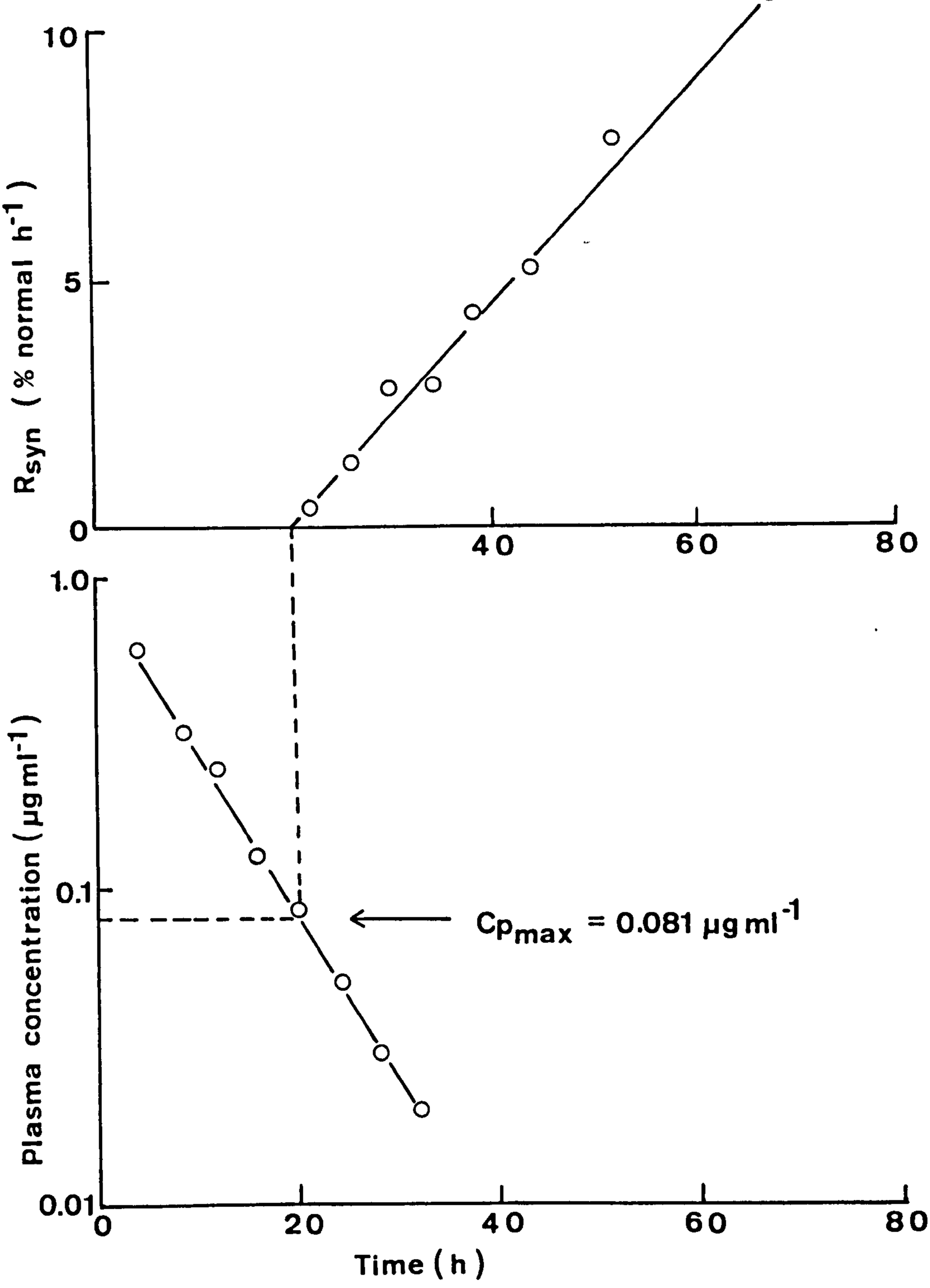


TABLE 3.4 The minimum plasma concentrations of racemic warfarin, R(+) warfarin and S(-) warfarin that completely inhibit clotting factor synthesis (Cp_{max}) in the rabbit.

	Dose (mg kg^{-1})	Cp_{max} (µg ml^{-1})
Racemic warfarin	6.30	0.103 ± 0.031
Racemic warfarin	0.63	0.103 ± 0.098
R(+) Warfarin	0.63	0.099 ± 0.018
S(-) Warfarin	0.63	⩽ 0.050

Values show mean ± standard error (n = 4)

3.5 Discussion

The pharmacological response to warfarin and its enantiomers in man and experimental animals is dependent on both the pharmacokinetics and the pharmacodynamics of these anticoagulant compounds. Consequently it is important to determine the relative contributions of the pharmacokinetics and the pharmacodynamics to the pharmacological effect observed *in vivo*.

Pharmacokinetics of Racemic Warfarin and its Enantiomers

After intravenous administration of single equimolar doses of racemic, R(+) and S(-) warfarin, the plasma concentrations of each anticoagulant declined mono-exponentially; indicating single compartment pharmacokinetics for all three forms of warfarin.

As racemic warfarin consists of equal amounts of R(+) and S(-) warfarin, it seems reasonable to suggest that the pharmacokinetic parameters calculated for racemic warfarin should lie between those obtained for the enantiomers of warfarin, since there is no pronounced pharmacokinetic interaction between single doses of R(+) and S(-) warfarin (Levy *et al.*, 1978). This was true for mean plasma clearance and apparent volume of distribution following both doses of racemic warfarin. Furthermore the mean plasma half-life after the higher dose of warfarin falls between that for R(+) and S(-) warfarin, however after the ten-fold lower dose, the mean plasma half-life was greater than that for both enantiomers. This may be explained by the considerable variation in plasma half-lives of racemic warfarin for the rabbits in this group (4.33 to 14.81 h).

The plasma half-life of racemic warfarin in the rabbit (5.6 h) is similar to that observed in the rat (5.3 h; Yacobi *et al.*, 1974)

but is considerably shorter than that reported for man (15 to 80 h, O'Reilly et al., 1963; O'Reilly and Aggeler, 1968). The half-life of S(-) warfarin (4.3 h) was determined to be shorter than that of R(+) warfarin (6.4 h). As plasma half-life is a hybrid term dependent upon both plasma clearance and apparent volume of distribution, the differences in the half-lives of the enantiomers of warfarin can be explained by these other pharmacokinetic parameters. The plasma clearance of S(-) warfarin is almost 4 times that of R(+) warfarin and the volume of distribution of S(-) warfarin is more than twice that of R(+) warfarin.

Previous studies in man (O'Reilly, 1982) and the rat (Yacobi et al., 1984) have failed to reveal any difference in the apparent volume of distribution of the enantiomers of warfarin. The dissimilarity observed in the present study may be a consequence of a difference in the affinity of plasma proteins for the enantiomers of warfarin. The binding of S(-) warfarin to albumin is significantly greater than that of R(+) warfarin in both man (Sellers and Koch-Weser, 1975) and the rat (Yacobi and Levy, 1977).

The present study illustrates that the pharmacokinetics of racemic warfarin in the rabbit, are independent of dose over the ten-fold dose range investigated, which is consistent with a recent report in which no evidence of dose dependency could be found in an individual who had taken a massive (2000 mg) overdose of warfarin (Hackett et al., 1985).

Pharmacodynamics of Racemic Warfarin and its Enantiomers

Complete inhibition of clotting factor synthesis followed by the recovery of P.C.A. without recourse to vitamin K_1 administration was achieved over a ten-fold dose range after single intravenous doses

of racemic warfarin. Following equimolar doses of racemic, R(+) and S(-) warfarin, P.C.A. recovered more rapidly after S(-) warfarin. This illustrates the shorter duration of action of S(-) warfarin when compared to racemic and R(+) warfarin.

As a consequence of the lack of relationship between P.C.A. and the plasma concentration of warfarin, R_{syn} was used as the pharmacological end point most suitable to investigate the pharmacology of racemic warfarin and its enantiomers. Following administration of all three forms of warfarin, R_{syn} recovered mono-exponentially, and in the case of racemic warfarin, recovered at a rate independent of dose. Furthermore the duration of action, as determined by the rate of recovery of R_{syn} is shorter following S(-) warfarin than after racemic or R(+) warfarin.

Relationship Between the Pharmacokinetics and Pharmacodynamics of Racemic Warfarin and its Enantiomers

The duration of pharmacological response to a drug is a function of receptor interaction and elimination of the drug from the body. In an attempt to determine the relative contributions of these two processes to the pharmacological effect of warfarin and its enantiomers, m, the slope of the concentration - response curve for these anticoagulants *in vivo*, have been calculated. The equation used to determine m, relates the rate of decline of anticoagulant effect (ΔI) to the rate of elimination of anticoagulant from plasma (k).

There was no significant difference in the m values obtained for racemic, R(+) and S(-) warfarin. This is consistent with the three forms of warfarin acting at the same receptor, thought to be the enzyme vitamin K epoxide reductase (Bell, 1978). Using the animal model developed in

the present study, m values have been determined for the potent and long acting anticoagulants brodifacoum and difenacoum (Breckenridge et al., 1985). These are not significantly different to those determined in the present study, thus indicating that these rodenticides act at the same receptor as warfarin.

The potency of S(-) warfarin has been determined to be greater than that of R(+) warfarin in man (O'Reilly, 1974a; Hewick and McEwen, 1973; Wingard et al., 1978). The S enantiomer is thought to be approximately 3 times more potent than R(+) warfarin. Similarly, in the rat, S(-) warfarin has been shown to be 5 to 7 times more potent than R(+) warfarin (Breckenridge and Orme, 1972; Yacobi and Levy, 1974). By measuring the minimum plasma concentration of anticoagulant required to completely inhibit clotting factor synthesis (Cp_{max}) in the rabbit, the greater potency of S(-) warfarin over R(+) warfarin has been determined. However, due to a lack of sensitivity of the assay (<50 ng ml^{-1}), the exact potency of S(-) warfarin could not be determined. The potency of R(+) warfarin was found to be very similar to that of racemic warfarin. The graphical method used to determine the relative potencies makes the fundamental assumption that there is simple equilibrium of warfarin and its enantiomers between plasma and receptor.

Takada and Levy (1979) determined that the hepatic uptake of warfarin is concentration-dependent in the rat, and that this may be related to dose-dependent saturation of the enzymes involved in the metabolism of warfarin. Covell et al., (1983) suggested that at least two different hepatic tissues exchange warfarin with plasma. One exhibits Michaelis-Menten saturation kinetics, while the second shows linear exchange kinetics with free plasma warfarin, and it is this association which leads to the metabolism of warfarin.

Thus determination of the minimum hepatic concentrations of warfarin and is enantiomers required to completely inhibit clotting factor synthesis would provide a more accurate measure of potency.

3.6 Conclusions

An animal model has been developed in which the pharmacokinetics and the pharmacodynamics of warfarin and its enantiomers were investigated and compared. Using mathematical models, which were shown to be suitable for the investigation of warfarin over a ten-fold dose range, the relative contributions of the pharmacokinetics and the pharmacodynamics to the overall pharmacological effect of this anticoagulant, and its enantiomers were determined.

Despite its shorter half-life, S(-) warfarin was shown to be more potent than R(+) warfarin in the rabbit..

The model developed in this study for warfarin can be usefully applied to investigate the pharmacology of other 4-hydroxycoumarin anticoagulants, especially the novel compounds, brodifacoum and difenacoum, since ingestion of these anticoagulant compounds leads to a life threatening degree of hypoprothrombinaemia.

CHAPTER 4

INVESTIGATION OF THE ENHANCED HYPOPROTHROMBINAEMIC RESPONSE TO WARFARIN IN THYROTOXIC PATIENTS

4.1 Introduction

The pharmacological effects of warfarin and related 4-hydroxy-coumarin anticoagulants can be altered in a number of diseased states. Severe hypoalbuminaemia (Lewis et al., 1967), congestive heart failure (Killip and Payne, 1960), acute viral hepatitis (Kliesch et al., 1960), and nephrotic syndrome (Ganeval et al., 1986) have all been shown to affect the daily dose of oral anticoagulant required to maintain a safe degree of anticoagulation in an individual.

Individuals suffering from hyperthyroidism show a high incidence of atrial fibrillation. This is associated with a risk of systemmic embolism, and for this reason they require anticoagulant therapy.

There are a number of reports of a potentiation of the hypoprothrombinaemic effect of several anticoagulants in the hyperthyroid state (Walters, 1963; Schrogie and Solomon, 1967; McIntosh et al., 1970). Furthermore, the administration of the thyroid analogue dextrothyroxine has been shown to enhance the anticoagulant effect of warfarin in both man (Owens et al., 1962) and the dog (Weintraub et al., 1973).

The mechanism for this altered responsiveness, both in hyperthyroidism and after the administration of dextrothyroxine, has not yet been defined. There are, however, many conflicting reports on the effect of the hypermetabolic state on both the pharmacokinetics of 4-hydroxycoumarin anticoagulants (Schrogie and Solomon, 1967; McIntosh et al., 1970), and on the activity of individual clotting factors (Egeberg, 1963; Loeliger et al., 1964; Simone et al., 1965). The different observations made by separate groups are most likely due to differences in the severity of the hyperthyroidism in the individuals studied. These case reports have appeared throughout the past 20 years and there is no measure of thyroid function which is common to

all the studies. It is therefore almost impossible to relate the degree of thyrotoxicosis to the pharmacological effect observed.

The aim of this study was to investigate both the pharmacokinetics and the pharmacodynamics of warfarin in patients who are initially thyrotoxic and then, after treatment, euthyroid, and also to apply the mathematical model developed for warfarin in the rabbit (Chapter 3), in order to understand the mechanism by which hyperthyroidism produces an enhanced pharmacological effect of warfarin.

4.2 Plan of Study

Five female patients with hyperthyroidism gave informed consent to take part in the study. They were all in normal sinus rhythm and there was no clinical evidence of systemic embolisation. Thyrotoxicosis was confirmed in all patients by the thyroid function tests described in Table 4.1. None of the patients were taking any drugs known to significantly affect the pharmacological response to warfarin or had had recent gastro-intestinal bleeding. The five patients were studied for their coagulation response to warfarin in the thyrotoxic state and again in the euthyroid state after effective treatment with [^{131}I].

Immediately prior to the oral administration of warfarin (0.14 mg kg^{-1}) at t = 0, control venous blood samples were collected for the determination of prothrombin ratio (PTR), partial thromboplastin time (PTT) and the activity of clotting factors II, VII, VIII, IX and X. Subsequent blood samples were collected at t = 6 h and then daily for the following 7 days, for the determination of plasma warfarin concentrations and the previously mentioned coagulation parameters. Further blood samples were taken at t = 2 h and t = 4 h, for the determination of plasma warfarin concentrations. Blood taken for the determination of plasma warfarin was collected into heparinised blood tubes, the plasma separated by centrifugation (2000 x g; 20 min) and stored frozen (-20°C) until required for analysis as described in section 2.3.1.

The following tests were carried out at the Department of Medicine, Royal Infirmary, Edinburgh: Quick one-stage prothrombin time, partial thromboplastin time with kaolin, and all assays for individual clotting factors except for prothrombin (Jobin and Esnouf, 1966) were

TABLE 4.1 Thyroid function tests used to confirm thyrotoxicosis

1. Serum thyroxine (T_4) and triiodothyronine (T_3) concentrations.

2. Free thyroxine index

3. Serum autoantibodies

4. Serum antibodies to thyroid stimulating hormone

5. Serum thyrotrophin releasing hormone

6. Uptake of [^{131}I]

carried out according to one-stage techniques, by the methods of Austen and Rhymes (1975).

Pharmacokinetic parameters were calculated from the plasma concentration-time data as described in section 2.4. Results are expressed as mean ± standard deviation of the mean (mean ± s.d.). Levels of significance were determined using the paired Student's t test.

4.3 Results

Pharmacokinetics of Warfarin in Thyrotoxicosis

Following a single oral dose of warfarin (0.14 mg kg^{-1}), plasma concentrations declined mono-exponentially in all five individuals in both the thyrotoxic and euthyroid states after the initial distribution phase (Figure 4.1). Table 4.2 illustrates the individual and the mean data obtained for the pharmacokinetic parameters calculated in the present study. The mean data for the four individuals excluding E.M., showed no significant change in any of the parameters between the thyrotoxic and the euthyroid states.

However E.M. displayed marked differences in all four pharmacokinetic parameters of warfarin. The area under the curve (AUC) was found to be 3 times greater in the euthyroid state (29.6 µg ml^{-1} h) than in the thyrotoxic state (94.7 µg ml^{-1} h), whereas the apparent volume of distribution was more than two-fold greater in the hyperthyroid state (0.231 l kg^{-1}) than in the normal state (0.096 l kg^{-1}). Plasma clearance of warfarin was three-fold greater in the hypermetabolic state (0.0788 ml min^{-1}) than in the euthyroid state (0.0246 ml min^{-1}).

Pharmacodynamics of Warfarin in Thyrotoxicosis

All five patients displayed an increased pharmacological effect as determined by PTR after a single oral dose of warfarin (0.14 mgkg^{-1}) in the thyrotoxic state when compared to the euthyroid state (Figure 4.2). The difference achieved maximal statistical significance ($P < 0.01$) on the second day following warfarin administration (Figure 4.3). One individual (E.M.) displayed an even greater potentiation

FIGURE 4.1 Individual plasma concentration of warfarin vs. time data in patients following oral administration of 0.14 mg kg^{-1} warfarin in both the thyrotoxic (★) and the euthyroid (●) state.

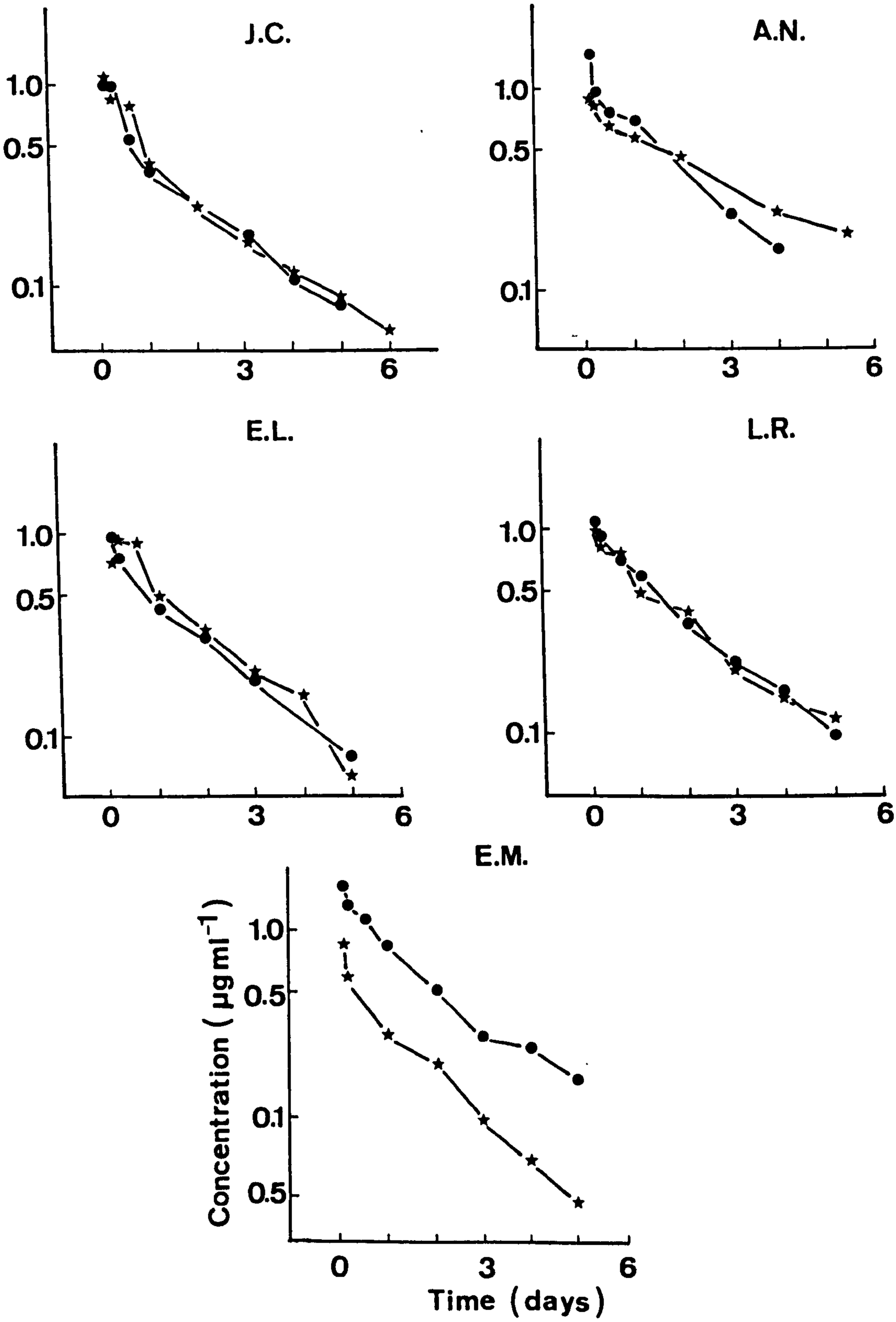


TABLE 4.2 Pharmacokinetic parameters of warfarin in patients in the thyrotoxic and euthyroid state.

Patient		AUC ($\mu g\ ml^{-1}\ h$)	$t_{\frac{1}{2}}$ (h)	Cl_p ($ml\ min^{-1}\ kg^{-1}$)	V_d ($l\ kg^{-1}$)
JC	T	44.6	46.2	0.0523	0.213
	E	43.9	39.2	0.0531	0.184
AN	T	89.1	60.3	0.0262	0.139
	E	65.5	38.5	0.0326	0.121
EL	T	46.9	36.1	0.0497	0.159
	E	44.3	36.5	0.0527	0.170
LR	T	56.0	43.3	0.0417	0.160
	E	54.8	40.8	0.0426	0.153
EM	T	29.6	33.2	0.0788	0.231
	E	94.7	44.1	0.0246	0.096
mean	T	58.4	46.5	0.042	0.167
$\pm$ s.d.		21.2	10.2	0.012	0.032
mean	E	52.1	38.8	0.046	0.157
$\pm$ s.d.		10.2	1.8	0.008	0.027

T - thyrotoxic

E - euthyroid

mean - calculated excluding EM data

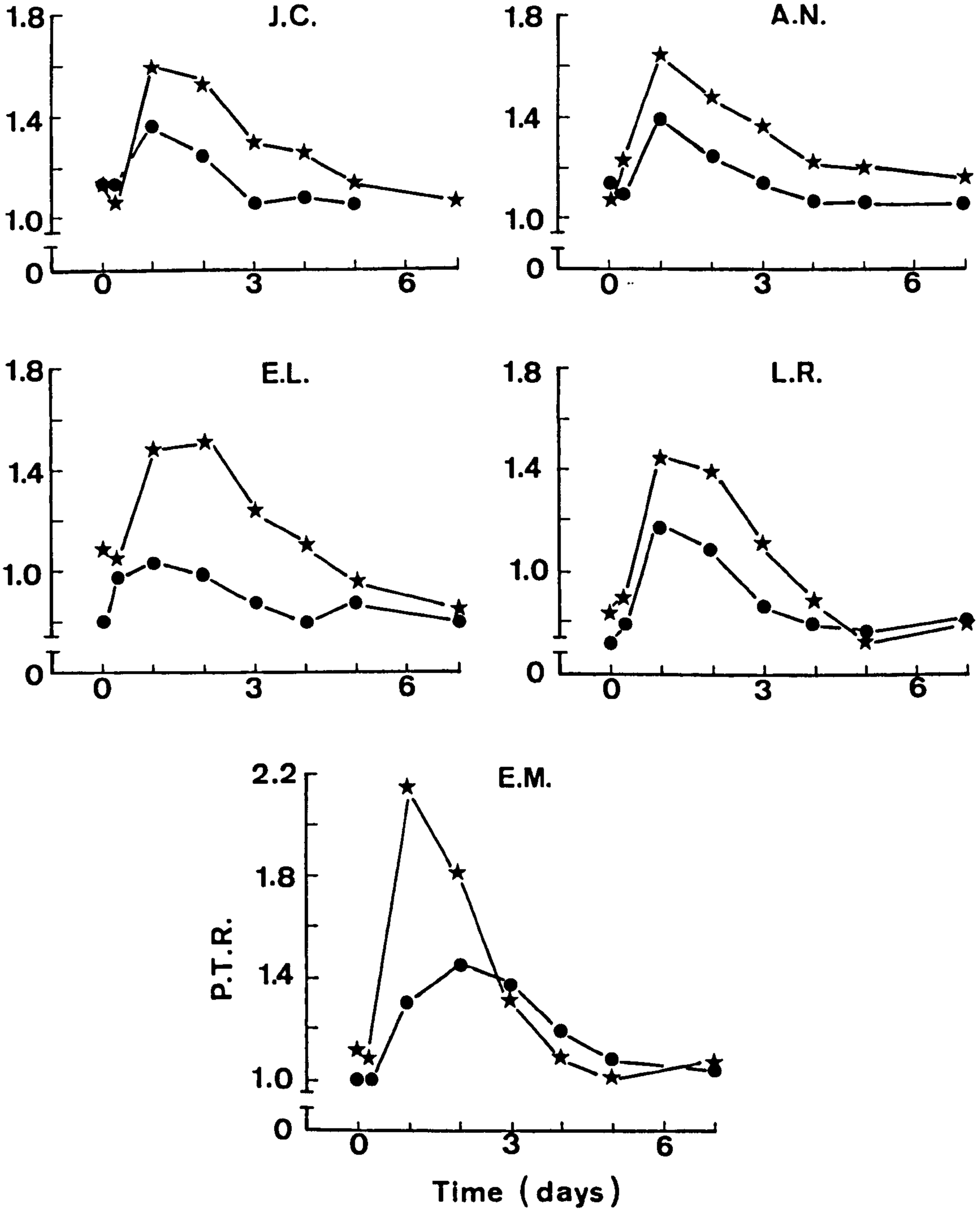
AUC - area under the curve

$t_{\frac{1}{2}}$ - plasma half-life

Cl_p - plasma clearance

V_d - apparent volume of distribution

FIGURE 4.2 Individual prothrombin ratio (P.T.R.) vs. time data in patients following oral administration of 0.14 mg kg^{-1} warfarin in both the thyrotoxic (★) and the euthyroid (●) state.



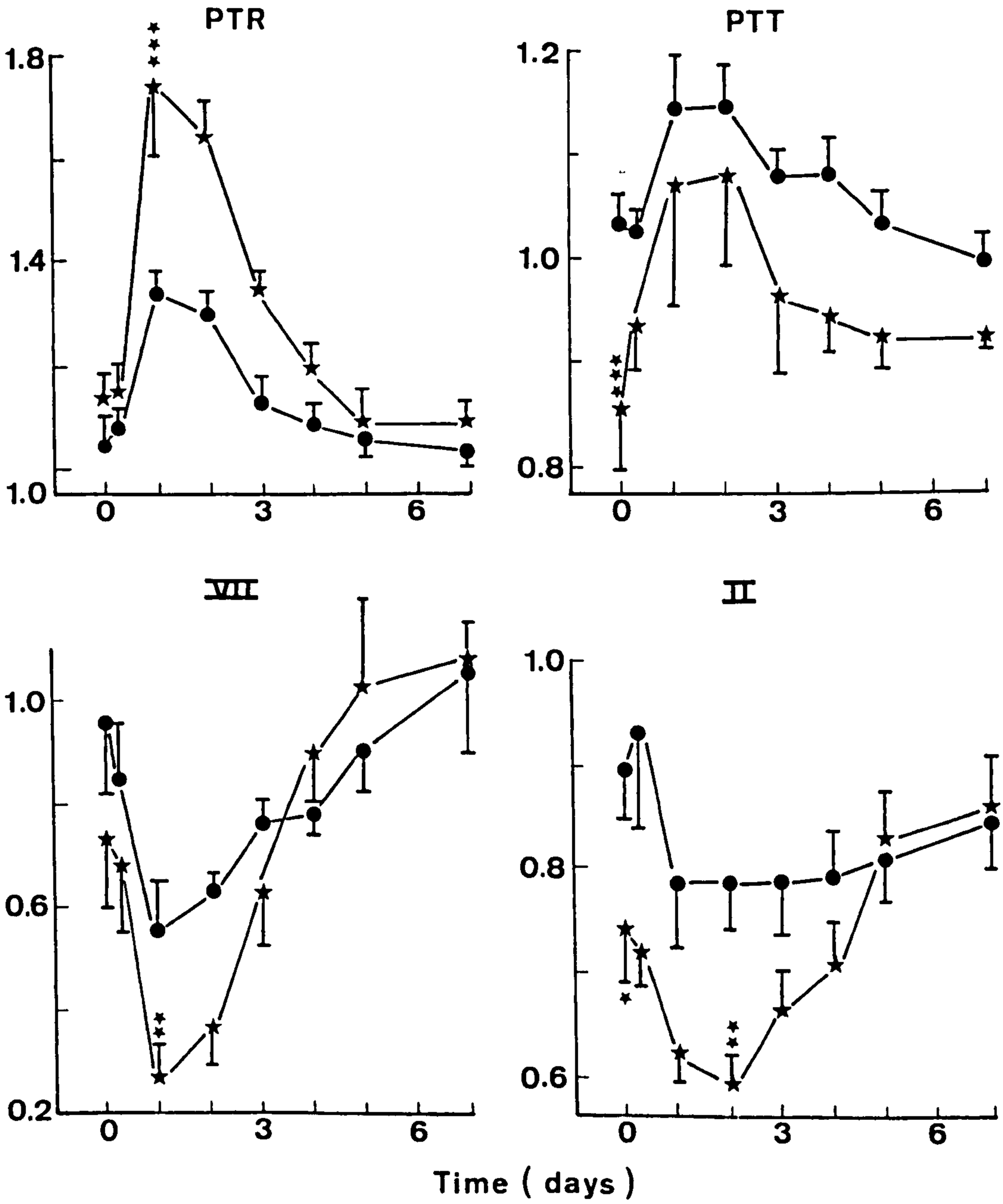
of the pharmacological effect in the hypermetabolic state, although the effect in the euthyroid state was very similar to that in the other four patients.

The mean data obtained for the activity of factor II and factor VII is illustrated in Figure 4.3. Following warfarin administration, the maximum depression of both factors II and VII were significantly ($P < 0.02$) greater in the hyperthyroid state than in the euthyroid state. The exaggerated fall in factor VII activity was coincidental with the prolongation of PTR in the hypermetabolic state. Furthermore, the mean basal factor II activity was significantly ($P < 0.05$) lower in hyperthyroidism than in the control state.

Even though basal PTT values were significantly ($P < 0.01$) lower in the hyperthyroid state than in the euthyroid state, following warfarin administration, there was a greater increase in PTT in the hypermetabolic state than in the euthyroid state (Figure 4.3).

There was no significant difference in the activity of factors VIII, IX and X at any of the time points investigated following warfarin, between the hyperthyroid and euthyroid states.

FIGURE 4.3 Prothrombin ratio (PTR), partial thromboplastin time (PTT), activity of factor II and activity of factor VII vs. time in patients following oral administration of 0.14 mg kg^{-1} warfarin in both the thyrotoxic (★) and the euthyroid (●) state.
Results are expressed as means (n = 5) and vertical bars show s.e. mean.
★, ★★, ★★★, significantly different from euthyroid at $P < 0.05$, $P < 0.02$ and $P < 0.01$ respectively.



4.4 Discussion

The aim of this study was to determine the relationship between the pharmacokinetics and the pharmacodynamics of warfarin in a small group of patients with thyrotoxicosis.

Pharmacokinetics of Warfarin in Thyrotoxicosis

There is little information in the literature concerned with the effect of thyrotoxicosis on the pharmacokinetics of warfarin. McIntosh and co-workers (1970) investigated both the pharmacokinetics and the pharmacological effect of warfarin in both the thyrotoxic and euthyroid state. They concluded that the enhanced responsiveness to warfarin in the hypermetabolic state was associated in some way with a decreased plasma half-life of the drug. In addition to this finding being inadequate to explain the increased pharmacological effect, the plasma half-lives determined once the patients were euthyroid are much greater (126 ± 29 h) than those commonly accepted for warfarin (15 to 80h; O'Reilly et al., 1963; O'Reilly and Aggeler, 1968; O'Reilly, 1982). A lack of specificity of the fluorimetric assay used to determine the plasma warfarin concentrations may explain the abnormally long half-lives obtained in that study. The plasma half-lives of warfarin determined in the present study in both the diseased and the control state, by HPLC with UV detection, fall within the normally accepted range. Although the general trend was a decreased plasma half-life in the euthyroid state, this difference was not statistically significant.

Serum albumin concentrations have been shown to be markedly reduced in some cases of hyperthyroidism (Kimberg et al., 1971). Feely and co-workers (1981a) suggested this as an explanation of decreased plasma protein binding of warfarin in hyperthyroidism. Warfarin has a high

degree of plasma protein binding (O'Reilly, 1980a) and as a consequence small changes in plasma albumin concentrations may increase the amount of warfarin available for biotransformation due to the restrictive nature of the elimination of warfarin. An increased plasma clearance would therefore be seen, however this effect would only be transient, since the free warfarin would establish a new equilibrium with bound warfarin. The plasma clearance values obtained in the present study in the thyrotoxic state are not significantly different to those obtained in the euthyroid state, except for the individual (E.M.) who displayed a three-fold increase in the plasma clearance of warfarin in the thyrotoxic state when compared to the euthyroid state. A decreased albumin concentration followed by an increased free fraction of warfarin would not account for the increased anticoagulant effect in thyrotoxicosis in the clinical situation as these patients would be on steady state warfarin and in this situation responsiveness would be unchanged in the presence of altered binding (Koch-Weser and Sellers, 1971).

The apparent volume of distribution of warfarin determined for E.M. in the thyrotoxic state was more than twice that in the euthyroid state. This may suggest an increased ability of tissue proteins to bind warfarin in the hypermetabolic state, and may in part explain the greatly increased anticoagulant effect of warfarin in the hyperthyroid state in E.M. This suggestion is similar to that proposed by Solomon and Schrogie (1967) who explained the enhanced anticoagulant effect of warfarin in the hypermetabolic state by an increased affinity of hepatic receptor sites for the drug.

Pharmacodynamics of Warfarin in Thyrotoxicosis

The blood coagulation system is comprised of the intrinsic, the extrinsic and the common pathways, which interact with each other to

contribute to the maintenance of normal haemostasis. In the presence of warfarin, the synthesis of the vitamin K - dependent clotting factors II, VII, IX and X is inhibited (Jackson and Suttie, 1977). Three of these clotting factors II, VII, and X are involved in the extrinsic clotting system, whose activity is determined by the measurement of PTR. PTT determines the activity of factor IX which is involved in the intrinsic pathway.

Consistent with previous reports of an enhanced hypoprothrombinaemic effect of warfarin and related 4-hydroxycoumarin anticoagulants in thyrotoxicosis (McIntosh et al., 1970; Vagenakis et al., 1972; Self et al., 1976), the patients involved in the present study displayed increased PTR following warfarin in the thyrotoxic state. PTR is sensitive to changes in the activity of factor VII, which has the shortest half-life of all the clotting factors in man and is therefore the most sensitive to the effect of warfarin. Consistent with this, following warfarin administration the activity of factor VII decreased rapidly in both conditions in all five patients, and is coincidental with the increased values of PTR obtained in both the hyperthyroid and euthyroid state.

Significantly lower basal activity of factor II was observed in the hyperthyroid state when compared to the control situation. This has not previously been described, although Weintraub and co-workers (1973) reported that the degradation rate of factor II was increased by 40% in dogs given D-thyroxine. Evidence of an increased degradation of factor VII has been observed in hyperthyroidism in man (Loeliger et al., 1964).

In the present study, basal PTT was found to be significantly shortened in the thyrotoxic state when compared to the euthyroid state;

thus indicating an increased activity of the intrinsic pathway in the diseased state. This is consistent with the work of Simone and co-workers (1965) and may indicate an increased tendency for intravascular thrombosis in hyperthyroidism.

The Relationship Between the Pharmacokinetics and the Pharmacodynamics of Warfarin in Thyrotoxicosis

From the results obtained so far in this study, it appears that a pharmacodynamic difference in the thyrotoxic state is responsible for the increased pharmacological effect of warfarin. However to accurately distinguish between a pharmacokinetic or a pharmacodynamic effect, in vivo concentration-response curves have to be constructed.

As discussed in Chapter 3, there is no direct relationship between P.C.A. and plasma concentration of warfarin. However, if the pharmacological effect of warfarin is expressed in terms of the degree of inhibition of the rate of synthesis of P.C.A. (R_{syn}) then an essentially linear relationship exists (over the clinically significant range) between the intensity of effect and the log of the concentration of warfarin in plasma (Nagashima et al., 1969). This approach has been used in a number of studies to determine the potencies of the individual enantiomers of a number of 4-hydroxycoumarin anticoagulants in man and experimental animals (Breckenridge and Orme, 1972; Jähnchen et al., 1976). Furthermore, it has been used to determine the mechanism of the interaction of another drug with a coumarin anticoagulant (Jähnchen et al., 1973; Schmidt and Jähnchen, 1979).

The widespread use of this model to study the pharmacology of 4-hydroxycoumarin anticoagulants, and the fact that a slightly modified version of this model was shown to be ideal to investigate the pharmaco-

logical effect of warfarin over a ten-fold dose range in the rabbit (Chapter 3) justifies the use of this model in this clinical situation.

Thus the model was employed to distinguish between changes in the pharmacokinetics of warfarin and changes in the responsiveness of the clotting system induced by the hypermetabolic state.

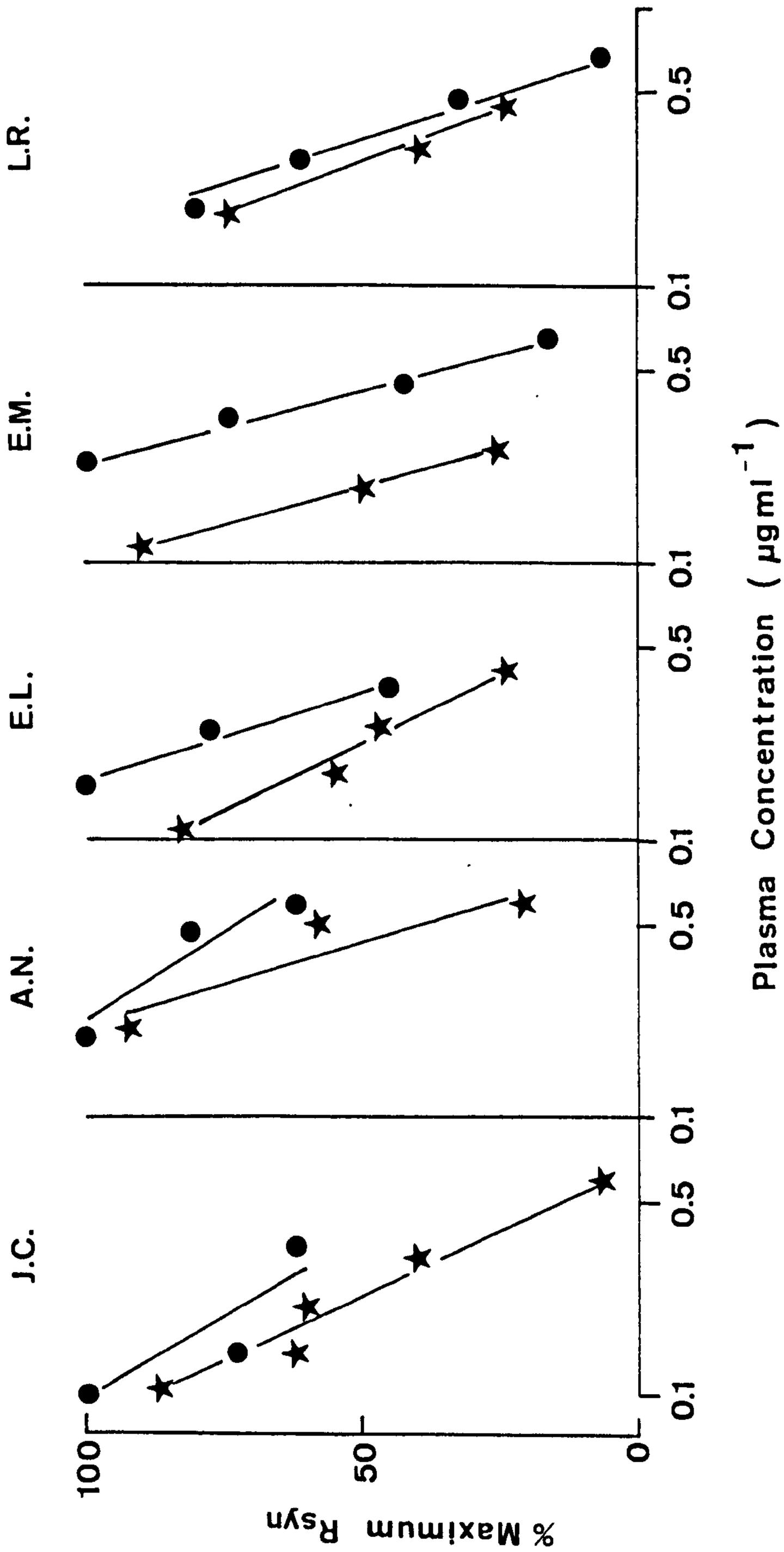
Figure 4.4 illustrates the % of maximum rate of synthesis of P.C.A. (R_{syn}) plotted against the log of the plasma concentration of warfarin for the five patients involved in the present study. R_{syn} was calculated as described in section 3.4. Concentration-response curves were obtained for all five individuals in both the thyrotoxic and euthyroid state.

The results obtained suggest an increased receptor sensitivity to warfarin in the thyrotoxic state in all five individuals since the concentration-response curves in the diseased state were always to the left of those determined in the control situation. One individual (E.M.) displayed a very pronounced shift of the concentration-response curve to the left in the thyrotoxic state. This indicates an even greater sensitivity to warfarin in this individual and is reflected by a greater pharmacological effect as determined by PTR.

The pharmacological analysis employed in the present study has enabled the relative contributions of the pharmacokinetics and the pharmacodynamics to the overall pharmacological effect of warfarin to be determined in the thyrotoxic and the euthyroid states. It appears that an apparent increase in receptor sensitivity to warfarin is responsible for the increased anticoagulant effect observed in the hypermetabolic state.

Many studies have investigated the effect of altered thyroid

FIGURE 4.4 % of maximum rate of synthesis of prothrombin complex activity (R_{syn}) vs. plasma concentration of warfarin in patients following oral administration of 0.14 mg kg^{-1} warfarin in both the thyrotoxic (★) and the euthyroid (●) state.


L.R.
E.M.
E.L.
A.N.
J.C.
0.5
0.1
0.5
0.1
0.5
0.1
0.5
0.1
Plasma Concentration ($\mu g ml^{-1}$)
100
50
0
% Maximum R_{syn}

state on the pharmacology of a variety of drugs. In contrast to the effect of hyperthyroidism on the pharmacological response to warfarin, pharmacokinetic changes rather than apparent changes in receptor sensitivity explain the altered responsiveness to many drugs including cardiac glycosides (Croxson and Ibbertson, 1975) and methimazole (Crooks et al., 1973) in the hypermetabolic state. Furtheremore, these drugs display a decreased effectiveness in the hypermetabolic state whereas this condition causes an increased hypoprothrombinaemic effect of warfarin.

Thyroid hormones are known to affect fundamental molecular processes resulting in alterations to many physiological functions including metabolic rate and cholesterol synthesis. A number of enzyme activities including glycerophosphate dehydrogenase, Na^+ K^+- ATPase and mixed function monooxygenases are increased in the presence of thyroid hormones. Thus the apparent increase in receptor sensitivity to warfarin in thyrotoxicosis may be due to elevated protein (clotting factor) turnover, that is, an increase in both the catabolism and the anabolism of clotting factors. This theory is substantiated by the observed effects of thyrotoxicosis on the activity of clotting factors II and VII in the present study, and also by the results of previous studies. An increased rate of degradation of factor II has been reported in man in the thyrotoxic state (Loeliger et al., 1964) and an increased rate of degradation of factor II has been reported in dogs receiving D-thyroxine (Weintraub et al., 1973).

4.5 Conclusions

The method of pharmacological analysis employed to investigate the pharmacological effect of warfarin and its enantiomers in the rabbit (Chapter 3) has allowed the pharmacokinetics and the pharmacodynamics of warfarin to be distinguished in patients before and after effective treatment of thyrotoxicosis.

There was no significant difference in the pharmacokinetics of a single oral dose of warfarin between the thyrotoxic and the euthyroid state. However, when plasma concentration of warfarin - response (as determined by % of maximum rate of synthesis of P.C.A.) curves were plotted in both the diseased and the control states, the curves produced in thyrotoxicosis were to the left of those in the euthyroid state in all five individuals studied.

Since thyroid hormones are known to increase the activity of a number of enzyme activities, it is proposed that the apparent increased receptor sensitivity to warfarin in the hypermetabolic state, is due to increased protein (clotting factor) turnover. The enhanced effect of warfarin on the activity of factor VII and the decreased basal activity of factor II in thyrotoxicosis in the present study, substantiates this proposal.

CHAPTER 5

INVESTIGATION OF A POSSIBLE STEREOSELECTIVE INTERACTION BETWEEN WARFARIN AND CIMETIDINE IN VOLUNTEERS

5.1 Introduction

Altered responsiveness to 4-hydroxycoumarin anticoagulants may have either a pharmacokinetic or pharmacodynamic basis. In Chapter 4 both the pharmacokinetics and the pharmacodynamics of a single dose of warfarin were investigated in patients to determine the underlying mechanism by which hyperthyroidism produces an enhanced anticoagulant effect of warfarin. The present chapter describes a study designed to determine the fundamental basis of the pharmacological interaction between warfarin and the H_2 antagonist, cimetidine.

Cimetidine is a very commonly prescribed drug and as a consequence, the potential for drug interactions is exceedingly high. Clinically relevant interactions of cimetidine with a wide range of drugs have been described (Somogyi and Gugler, 1982). Inhibition of metabolism has been shown to be the major cause of drug interactions with cimetidine. A large proportion of the drugs affected are those metabolised by the mixed function monooxygenase enzymes and include antipyrine (Staiger et al., 1981; Daneshmend et al., 1980), diazepam (Klotz and Reinmann, 1980), phenytoin (Neuvonen et al., 1981) and theophylline (Roberts et al., 1981). However, the reduced clearance of propranolol in the presence of cimetidine has been shown to be due to reduced liver blood flow (Feely et al., 1981b). Furthermore, the lack of response to ketoconazole when co-administered with cimetidine was attributed to reduced absorption of the antifungal agent in an elevated gastric pH (Van der Meer et al., 1980).

One of the first clinically important interactions with cimetidine was with warfarin. This was shown to result in increased plasma concentrations of the anticoagulant and a prolongation of pro-

thrombin time (Serlin et al., 1979; O'Reilly, 1984). The basis of this interaction is thought to involve inhibition of drug metabolism.

Warfarin is administered clinically as a racemic mixture of R(+) and S(-) warfarin. The mean potency of S(-) warfarin has been reported to be 3.8 (Breckenridge et al., 1974), 3.4 (O'Reilly, 1974) and 2.7 (Wingard et al., 1978) times that of R(+) warfarin in man.

Several drug interactions with warfarin have been shown to have a stereochemical basis. Perhaps the most widely publicised and documented interaction of warfarin is that with the anti-inflammatory drug phenylbutazone (Aggeler, 1967). A significant increase in the plasma clearance of R(+) warfarin and a significant decrease in the plasma clearance of S(-) warfarin was observed in the presence of phenylbutazone (Lewis et al., 1974). Banfield and co-workers (1983) determined this stereoselective effect to be due to a combination of a number of pharmacokinetic processes. Displacement of R(+) and S(-) warfarin from plasma albumin and inhibition of 6 and 7-hydroxylation of both enantiomers were observed in the presence of phenylbutazone.

O'Reilly (1976) reported that the anti-protozoal and bactericidal agent, metronidazole inhibits the elimination and enhances the anticoagulant effect of the S enantiomer but not of the R enantiomer. Similarly, the bactericidal agent cotrimoxazole has been shown to elevate the plasma concentration and the anticoagulant effect of S(-) warfarin but produced no change in these parameters for R(+) warfarin (O'Reilly, 1980b).

The aim of the present study was to investigate the possibility of a stereoselective interaction between warfarin and cimetidine in healthy volunteers.

5.2 Plan of Study

Seven healthy volunteers were involved in this study. Each individual gave informed consent and approval was obtained from the Mersey Regional Ethics Committee.

Volunteers took an oral dose (15 mg) of a single enantiomer of warfarin, each individual receiving both enantiomers of warfarin with or without cimetidine. The order of the four parts of the study was randomised for each volunteer. Cimetidine, given in a dose of 200 mg three times a day and 400 mg at night, was started 4 days before the administration of the warfarin and continued until the collection of the last blood sample.

The warfarin enantiomers (97% pure) were prepared by dissolving the solid in 0.5 M NaOH, adjusting the pH to 8.5 with 0.5 M HCl and making the final volume up to 10 ml per dose. Vitamin K_1 (Konakion$^{(R)}$, 20 mg), diluted in 0.9% w/v NaCl solution (20 ml) was given intravenously over 20 min, together with the warfarin to prevent any prolongation of the prothrombin time. If prolongation of the prothrombin time (>15 s) did occur, it was corrected with oral vitamin K_1 (10 mg).

Blood samples (10 ml) were collected into heparinised blood tubes at 0, 0.5, 1, 2, 4, 6, 8, 12, 24, 48, 72, 96, 120, 144, 168, 192, 216 and 240 h following warfarin administration. Plasma was obtained by centrifugation (2000 x g; 20 min) and stored frozen (-20^{o}C) until required for the determination of plasma concentrations of either R(+) or S(-) warfarin as described in section 2.3.1.

The effect of cimetidine on the plasma protein binding of warfarin was determined as described in section 2.3.8.

Results are expressed as mean $\pm$ standard deviation of mean

(mean ± s.d.). Levels of significance were determined using the Student's paired t-test.

5.3 Results

The mean data obtained for the pharmacokinetics of the enantiomers of warfarin in both the presence and the absence of cimetidine are illustrated in Table 5.1. One volunteer (JH) had to drop out of the study for personal reasons, and only data obtained for R(+) warfarin in this individual are included. There was a significant increase in both the area under the curve ($P < 0.01$) and the plasma half-life ($P < 0.002$) of R(+) warfarin in the presence of cimetidine, and also a significant ($P < 0.02$) decrease in plasma clearance. There was no change in the apparent volume of distribution of R(+) warfarin during cimetidine administration and all the pharmacokinetic parameters for S(-) warfarin remained unchanged during cimetidine therapy. The results obtained for a single volunteer (BH), which are typical, are illustrated in Figures 5.1 and 5.2. Table 5.2 illustrates the lack of effect of cimetidine (0.1 to 100 $\mu g\ ml^{-1}$) on the plasma protein binding of racemic warfarin *in vitro*.

TABLE 5.1 The effect of cimetidine on the pharmacokinetic parameters of the enantiomers of warfarin in volunteers

	R(+) Warfarin	R(+) Warfarin + cimetidine
AUC ($\mu g\ ml^{-1}\ h$)	96.1 ± 22.0	126.5 ± 14.3**
$t_{\frac{1}{2}}$ (h)	47.8 ± 5.4	57.8 ± 4.2***
Cl_p ($ml\ h^{-1}\ kg^{-1}$)	2.33 ± 0.52	0.70 ± 0.11*
V_d ($l\ kg^{-1}$)	0.16 ± 0.04	0.14 ± 0.01
	S(-) Warfarin	S(-) Warfarin + cimetidine
AUC ($\mu g\ ml^{-1}\ h$)	80.0 ± 43.7	81.1 ± 49.2
$t_{\frac{1}{2}}$ (h)	38.4 ± 13.8	37.7 ± 13.4
Cl_p ($ml\ h^{-1}\ kg^{-1}$)	3.12 ± 1.28	3.24 ± 1.43
V_d ($l\ kg^{-1}$)	0.15 ± 0.03	0.16 ± 0.03

Values show mean ± standard deviation of mean, n = 7 for R(+) Warfarin and n = 6 for S(-) Warfarin.

AUC - area under the curve

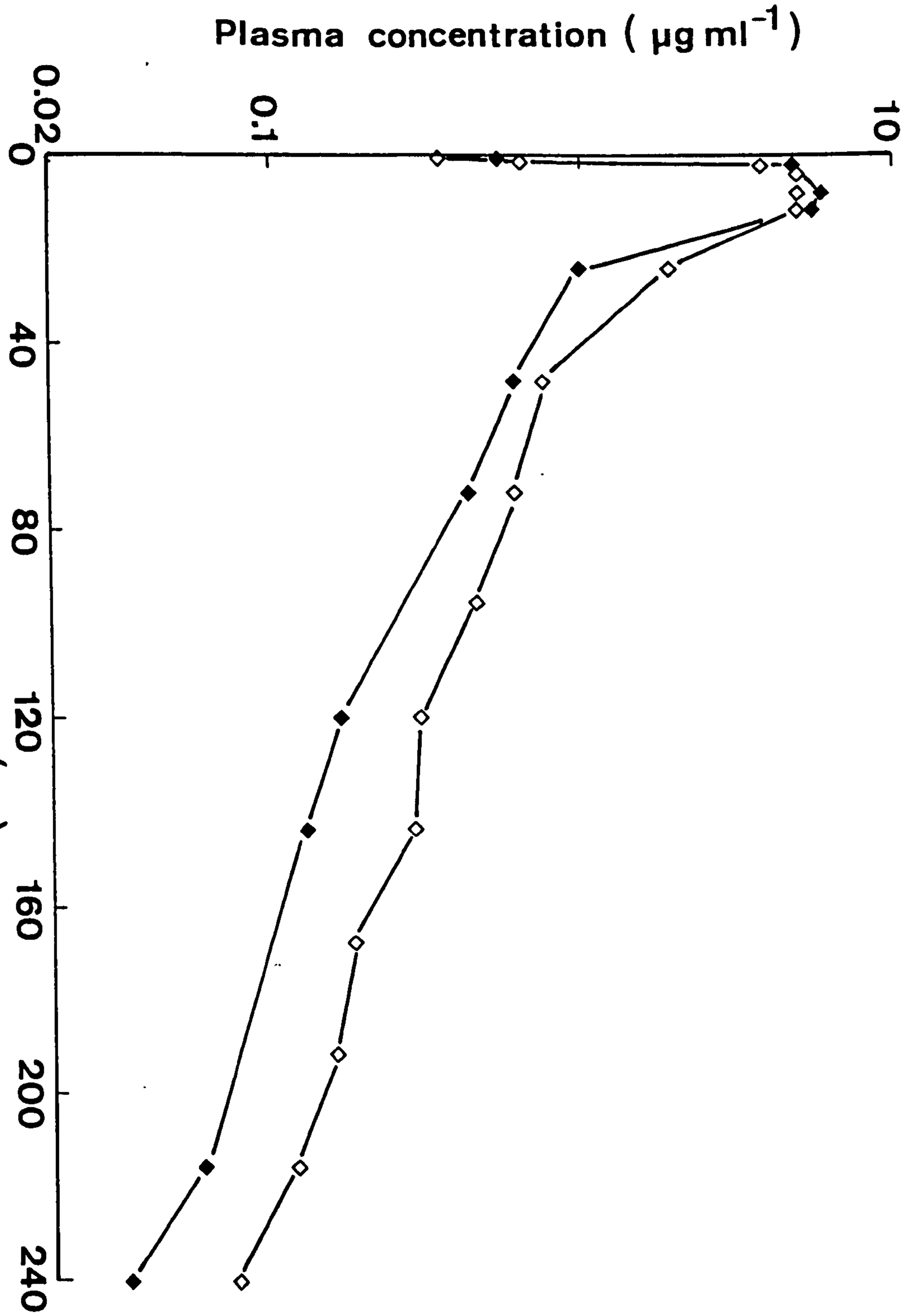
$t_{\frac{1}{2}}$ - plasma half-life

Cl_p - plasma clearance

V_d - apparent volume of distribution

*, **, *** Significantly different from S(-) Warfarin, $P < 0.02$, $P < 0.01$ and $P < 0.002$ respectively.

FIGURE 5.1 Plasma concentrations of R(+) warfarin vs. time in a single individual (BH) following oral administration of 15 mg R(+) warfarin with (◇) and without (◆) cimetidine.


Plasma concentration (μg ml^{-1})
0
0.02
0.1
1
0
40
80
120
160
200
240
Time (h)

142.

FIGURE 5.2 Plasma concentrations of S(-) warfarin vs. time in a single individual (BH) following oral administration of 15 mg S(-) warfarin with (O) and without (●) cimetidine.

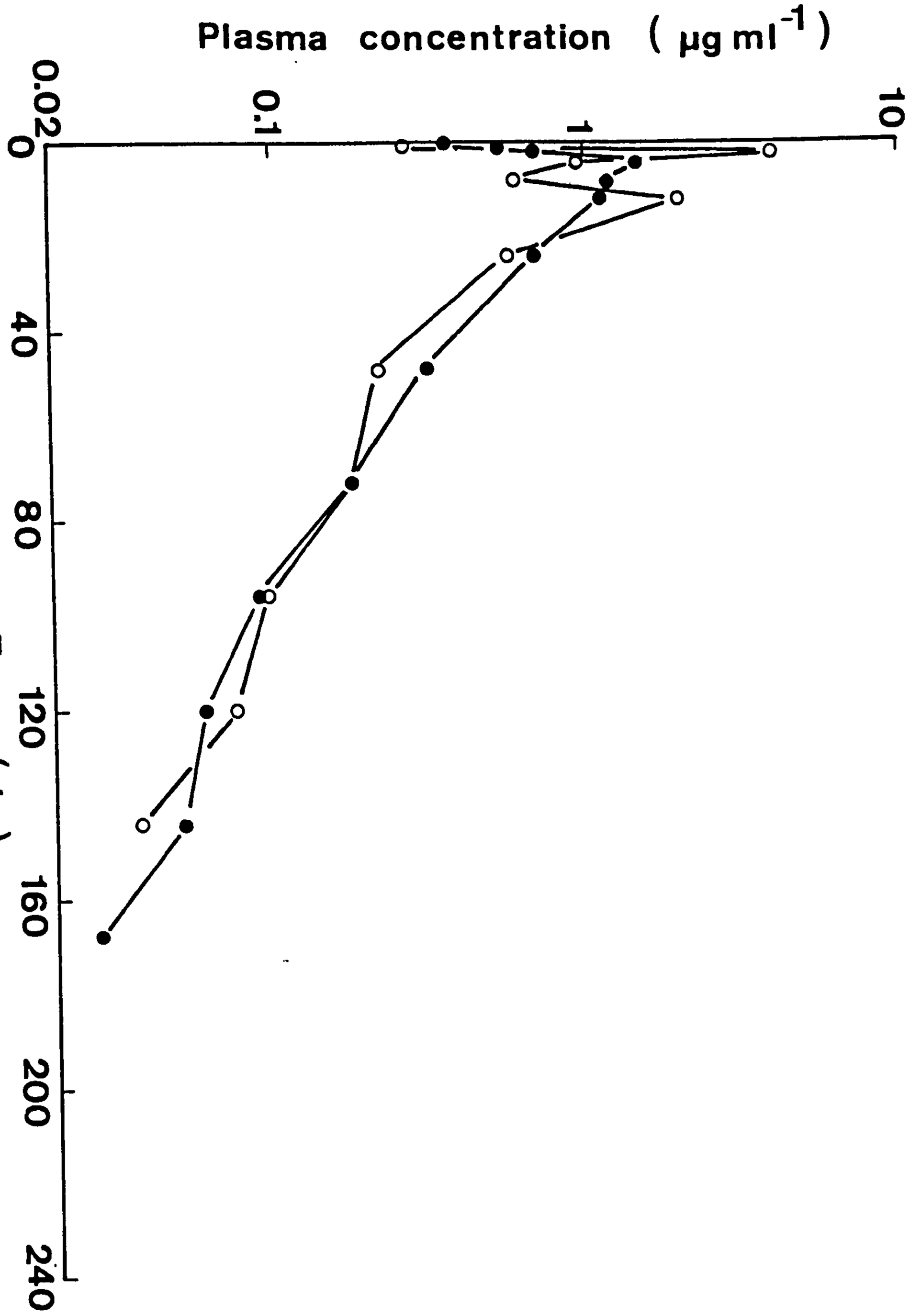

Plasma concentration (μg ml^{-1})
0
0.02
0.1
1
10
40
80
120
160
200
240
Time (h)

TABLE 5.2 Effect of cimetidine on plasma protein binding of racemic warfarin *in vitro* at 37°C, pH 7.4.

Concentration of cimetidine ($\mu g\ ml^{-1}$)	Free warfarin (%)
0	1.2
0.1	1.1
1.0	1.2
10	0.9
100	1.2

5.4 Discussion

The pharmacodynamic interaction between warfarin and cimetidine has been previously documented in volunteers (Serlin et al., 1979; O'Reilly, 1984) and in experimental animals (Serlin et al., 1980). Serlin and co-workers ascribed the enhanced hypoprothrombinaemic effect of warfarin in volunteers to an alteration in the pharmacokinetics of the anticoagulant during steady state cimetidine therapy. Therefore, the present study was solely concerned with the pharmacokinetics of the enantiomers of warfarin in the presence of cimetidine. The objective of the study was to investigate a possible stereoselective basis for this interaction.

The results obtained illustrate an alteration of the pharmacokinetics of R(+) warfarin in the presence of cimetidine. There was an increase in the area under the curve and the plasma half-life, and a decrease in the plasma clearance. By considering basic pharmacokinetic concepts, an explanation for these effects can be determined.

Absorption

Warfarin, when given in solution, displays a bioavailability of nearly 100% (Breckenridge and Orme, 1973). It has a pKa value of 4.7, and is therefore mostly in the unionised form at the range of pH values found in the stomach (1 to 3.5). Cimetidine causes decreased acid secretion and thus leads to an increase in gastric pH. However, there would have to be a substantial increase in gastric pH to cause any significant reduction in warfarin absorption. Furthermore, it is most unlikely that absorption of warfarin is a stereoselective process

Liver Blood Flow

Cimetidine has been shown to significantly reduce liver blood flow in healthy volunteers (Daneshmend et al., 1984) and has subsequently been shown to reduce the clearance of oral propranol$^{ol}_{\Lambda}$ (Feely et al., 1981b). However, unlike propranol$^{ol}_{\Lambda}$ warfarin is a drug with a low hepatic extraction ratio, and as a consequence changes in liver blood flow have little effect on the clearance of this oral anticoagulant. Furthermore, it would be very difficult to explain a stereoselective effect by a decrease in liver blood flow.

Plasma Protein Binding

Warfarin displays a high degree of plasma protein binding (99%) over the usual therapeutic dose range (O'Reilly, 1980a). Displacement of warfarin from plasma proteins by another drug will lead to a transient increase in clearance of the anticoagulant due to the fact that warfarin undergoes capacity limited, binding sensitive elimination. Cimetidine has a relatively low degree of plasma protein binding (18 to 26.3% bound at concentrations of 0.05 to 50 μg ml^{-1}; Taylor et al., 1978). Thus, the lack of effect of cimetidine on the binding of warfarin at concentrations 100 fold greater than those encountered in the therapeutic situation, is not unexpected. The decrease in the plasma clearance of R(+) warfarin in the presence of cimetidine is unlikely to be solely due to a change in the plasma protein binding of warfarin. If displacement is involved in this interaction, its effects are probably either masked or enhanced by a change in the metabolism of the enantiomers of warfarin.

Enzyme Inhibition

Cimetidine is known to be a potent inhibitor of drug metabolism

especially of those compounds metabolised by the hepatic mixed function monooxygenase enzymes (Somogyi and Gugler, 1982). Serlin and co-workers (1979) explained the enhanced hypoprothrombinaemic effect of warfarin in the presence of cimetidine by inhibition of metabolism of the anticoagulant. In the present study, although a change in the pharmacokinetics of R(+) warfarin was observed, there was no change in the pharmacokinetics of S(-) warfarin during cimetidine administration. Thus it appears that the metabolism of R(+) warfarin is inhibited whereas that of S(-) warfarin is unaffected by cimetidine administration.

Using liver microsomes from rats pretreated with various inducers of cytochrome P_{450}, Rendic and co-workers (1979) demonstrated a ligand interaction between cimetidine and cytochrome P_{450}. It has been suggested that cimetidine interacts with the haem iron of cytochrome P_{450}, both with its imidazole and cyano co-ordinating groups (Rendic *et al.*, 1983). Furthermore, it has been suggested that the effect of cimetidine on oxidative metabolism is more marked in individuals already enzyme induced.

In an *in vitro* study involving human liver microsomes, each enantiomer of warfarin was shown to undergo at least six separate cytochrome P_{450} mediated reactions (Kaminsky *et al.*, 1984) (Table 5.3). The major metabolites of R(+) and S(-) warfarin were 6-hydroxy and 7-hydroxywarfarin respectively. The results obtained in the present study suggest that cimetidine inhibits one of the pathways upon which R(+) warfarin relies heavily for its metabolism, but which is only a minor pathway for the metabolism of S(-) warfarin. This pathway is probably that of 6-hydroxylation. *In vitro* work, involving human liver biopsy samples, has shown that cimetidine has

TABLE 5.3 Rate of formation of the matabolites from R(+) and S(-) warfarin by hepatic microsomal preparations from human subjects.

Warfarin metabolite	Rate of metabolite formation (nmol product nmol P_{450}^{-1} min^{-1})	
	R(+) Warfarin	S(-) Warfarin
dehydrowarfarin	22	29
4'-hydroxywarfarin	17	32*
6-hydroxywarfarin	111*	38
7-hydroxywarfarin	44	113*
8-hydroxywarfarin	46*	1
10-hydroxywarfarin	40*	9

Values show means (N = 33)

* Significantly higher than rate from opposite enantiomer,

$P < 0.05$

(from Kaminsky et al., 1984)

the ability to inhibit the hydroxylation of benz(a)pyrene and coumarin, as well as the O-deethylation of 7-ethoxycoumarin. Since hydroxylation of benz(a)pyrene was inhibited most, it was suggested that only certain forms of cytochrome P_{450} are affected by cimetidine (Puurunen et al., 1980). This is consistent with the findings of the present study, which indicate that only the single isozyme of cytochrome P_{450}, P_{450_7} is inhibited by cimetidine. Warfarin is a useful probe drug for the investigation of multiple forms of cytochrome P_{450}, since it forms a wide range of metabolites each thought to be produced by a different isozyme of cytochrome P_{450}.

5.5 Conclusion

The stereoselective pharmacokinetic interaction between the R enantiomer of warfarin and cimetidine has been demonstrated in this study. The mechanism of action of this interaction appears to be inhibition of drug oxidation leading to a regioselective effect on 6-hydroxylation as opposed to 7-hydroxylation.

This inhibition of metabolism of R(+) warfarin is in direct contrast to the results obtained in studies investigating the stereoselective interaction of warfarin with metronidazole (O'Reilly, 1976), cotrimoxazole (O'Reilly, 1980b), sulphinpyrazone (O'Reilly, 1982) and phenylbutazone (Banfield et al., 1983) which have shown an increase in the mean plasma half-life of S(-) warfarin due to decreased clearance.

It has been suggested that the clinical use of R(+) warfarin instead of racemic warfarin would prevent drug interactions (O'Reilly, 1976), however the results obtained in this study suggest that although this would prevent certain warfarin drug interactions, it would not prevent them all.

CHAPTER 6

A STUDY OF THE PHARMACOLOGICAL RELATIONSHIPS BETWEEN THE PHARMACOKINETICS, THE PHARMACODYNAMICS AND THE BIOCHEMICAL EFFECT OF WARFARIN IN PATIENTS ON STEADY STATE WARFARIN THERAPY

6.1 Introduction

4-hydroxycoumarin anticoagulants are the mainstay of therapy in the management of a variety of thrombo-embolic and vascular diseases. In 1982 it was estimated that approximately 50,000 people in this country were taking warfarin. In spite of its extensive use, one of the major problems associated with oral anticoagulant therapy is the inter-individual variation in pharmacological response (Breckenridge, 1977). This poses a problem in the prediction of a suitable dose for the initiation of warfarin therapy.

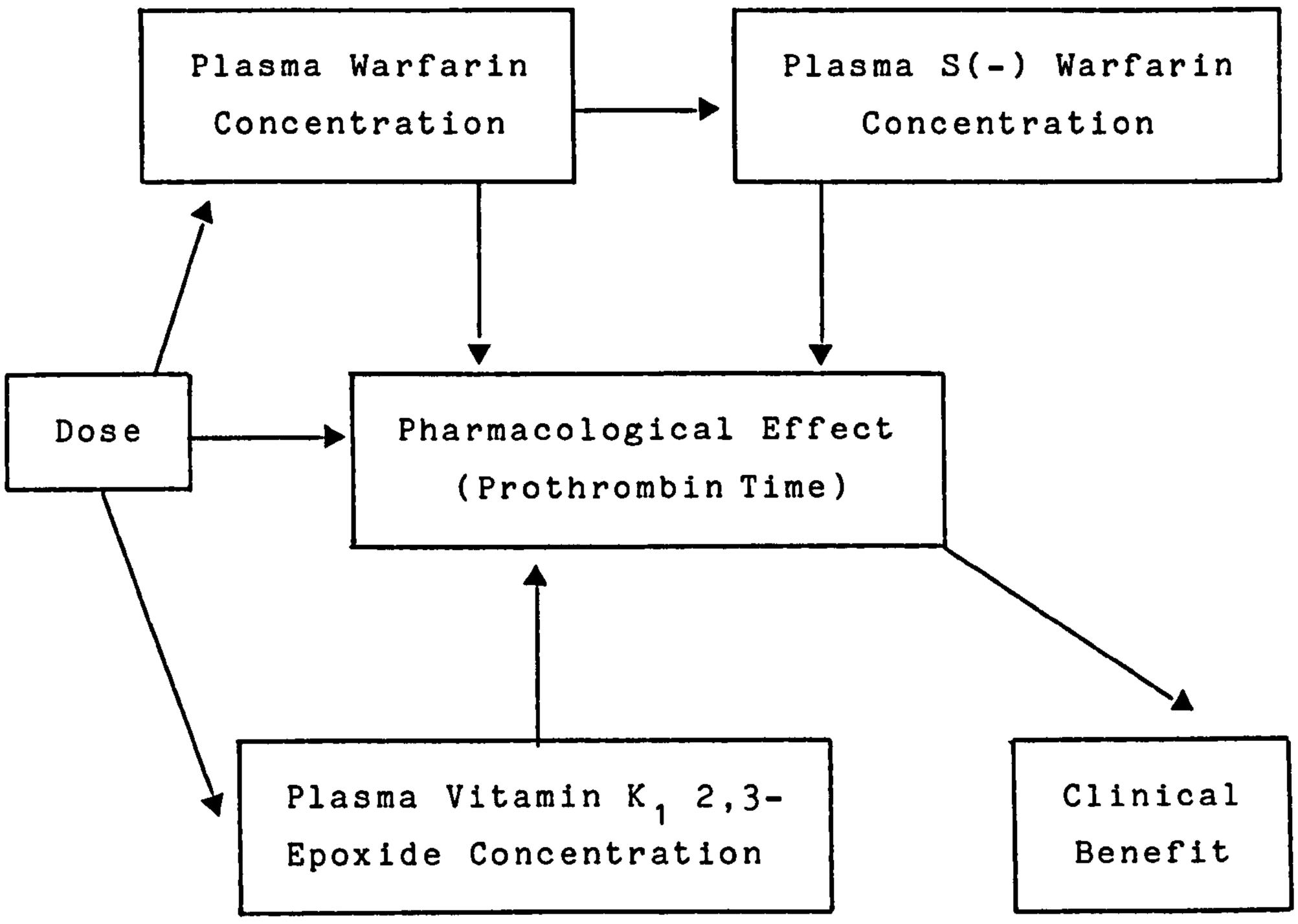
At present it is thought that warfarin and related oral anticoagulants interfere with clotting factor synthesis, by preventing the recycling of vitamin K_1 from the inactive metabolite, vitamin K_1 2,3-epoxide. The epoxide is produced during vitamin K-dependent γ-carboxylation of glutamic acid residues in clotting factor precursors (Larson et al., 1981), and under normal circumstances is converted back to vitamin K_1 by the enzyme vitamin K epoxide reductase (Willingham and Matschiner, 1974). The warfarin receptor is thought to be associated with this enzyme (Whitlon et al., 1978).

The purpose of the studies described in this chapter was to explore pharmacological relationships between changes in clotting factor synthesis and plasma warfarin concentrations, and thus provide some insight into factors which contribute to inter-individual variation in response to warfarin.

Figure 6.1 illustrates the nature of the investigations presented in this chapter. Following chronic administration of warfarin, the pharmacokinetics of the anticoagulant can be determined by the measurement of steady state plasma concentrations and the pharmacodynamics by measurement of prothrombin time. Consistent with the

FIGURE 6.1

Diagrammatic representation of the pharmacological relationships investigated in Chapter 6.



hypothesis that 4-hydroxycoumarin anticoagulants exert their pharmacological effect by inhibition of vitamin K epoxide reductase, an accumulation of vitamin K_1 2,3-epoxide has been detected in volunteers pretreated with warfarin (Shearer et al., 1973, 1977; Park et al., 1984). However, the epoxide is not detectable in the plasma of volunteers in the absence of 4-hydroxycoumarin anticoagulants (Park et al., 1984). Thus, the presence of vitamin K_1 2,3-epoxide in plasma can be used as a biochemical indicator of coumarin anticoagulation.

When administered clinically, warfarin consists of a racemic mixture of two optically active isomers, R(+) warfarin and S(-) warfarin. The enantiomers of warfarin show considerable differences in their pharmacokinetic and pharmacodynamic properties (Breckenridge et al., 1974; Wingard et al., 1978; Chan et al., 1984). The two initial studies described in this chapter were carried out to determine if any direct relationship exists between pharmacological effect as determined by prothrombin time and daily dose of racemic warfarin, plasma concentration of racemic warfarin and plasma concentration of S(-) warfarin in patients on steady state warfarin therapy.

The final study describes a more detailed investigation, again carried out in patients on steady state warfarin therapy, to determine if a direct relationship exists between plasma concentration of vitamin K_1 2,3-epoxide and prothrombin time. The aim of this study was to determine if plasma vitamin K_1 2,3-epoxide is a useful predictor of pharmacological response to warfarin.

It is important to undertake patient studies as well as volunteer studies since other drugs, disease and age may influence both the response to warfarin and its disposition. However, a disadvan-

tage of patient studies, is that the appropriate control experiments are not always possible.

6.2 Plan of Study

Liverpool Study

Forty-six outpatients taking warfarin were interviewed regarding age, daily dose of warfarin and other medication. Individual consent was obtained from each patient. 24 h following daily warfarin administration, a venous blood sample (10 ml) was collected from each patient into a heparinised blood tube. Plasma was obtained by centrifugation (2000 x g; 20 min) and stored frozen (-20°C) until required for the determination of plasma concentrations of racemic warfarin as described in section 2.3.1. At the same time venous blood samples were collected into a heparinised blood tube (5 ml) and into citrate (0.9 ml) for the determination of plasma concentrations of S(-) warfarin and the immediate determination of prothrombin time respectively. A radioimmunoassay described by Cook and co-workers (1979) was used to determine the plasma concentration of S(-) warfarin. Prothrombin time was determined as described in section 2.3.7.

Results are expressed as mean $\pm$ standard deviation of the mean (mean $\pm$ s.d.). r values were determined by linear regression analysis.

MRC Study

This study was carried out in conjunction with the MRC Epidemiology and Medical Care Unit, Northwick Park Hospital, Harrow, Middlesex. Sixty-nine outpatients on steady state warfarin therapy were involved in this investigation.

Prothrombin time was determined using rabbit brain thromboplastin (Diagen) at Northwick Park Hospital. Venous blood samples

(10 ml) were collected into non-heparinised blood tubes for the determination of plasma warfarin concentration as described in section 2.3.1. Results are expressed as mean ± standard deviation of the mean (mean ± s.d.). r values were determined by linear regression analysis.

Vitamin K_1 2,3-epoxide in anticoagulated patients

Eleven patients (four females) who were due to cease warfarin therapy, were involved in this study. Each individual gave informed consent and approval was obtained from the local ethics committees. Throughout the study all patients continued to take warfarin at the doses specified in Table 6.1.

On three successive days, 24 h after daily warfarin administration, a venous blood sample (10 ml) was collected from each individual into a heparinised blood tube. Plasma was obtained by centrifugation (2000 x g; 20 min) and stored frozen until required for the determination of plasma warfarin concentrations as described in section 2.3.1. At the same time, a venous blood sample (0.9 ml) was collected from each individual for the immediate determination of prothrombin time as described in section 2.3.7.

Each patient then received a single intravenous dose of vitamin K_1 (10 mg) diluted in 0.9% w/v NaCl solution (10 ml) over a period of 10 min. Blood samples (10 ml) were collected into heparinised blood tubes prior to vitamin K_1 administration and at 0.25, 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 24, 48, 72, 96, 120, 144 and 168 h after vitamin K_1 for the determination of plasma concentrations of vitamin K_1 2,3-epoxide as described in section 2.3.3.

Results are expressed as mean $\pm$ standard deviation of the mean (mean $\pm$ s.d.). r values were determined by linear regression analysis.

6.3 Results

Liverpool Study

The age of the patients involved in this study ranged from 30 to 80 years (57 $\pm$ 17 years). Thirty-seven patients were receiving other prescribed medication. Daily dose ranged from 2 to 13.5 mg (5.3 $\pm$ 2.6 mg) and the most commonly prescribed daily doses were 3, 4 and 5 mg.

The mean plasma concentrations of racemic and S(-) warfarin were 1.404 $\pm$ 0.561 and 0.676 $\pm$ 0.299 $\mu g\ ml^{-1}$ respectively. There was considerably inter-individual variation in the plasma concentrations of both racemic and S(-) warfarin at any given dose. For example, in patients receiving 4 mg of warfarin daily, plasma concentrations of racemic warfarin ranged from 1.143 to 2.183 $\mu g\ ml^{-1}$ (1.498 $\pm$ 0.414 $\mu g\ ml^{-1}$) and plasma concentrations of S(-) warfarin ranged from 0.395 to 1.000 $\mu g\ ml^{-1}$ (0.657 $\pm$ 0.208 $\mu g\ ml^{-1}$).

Prothrombin time ranged from 18 to 67 s (34.5 $\pm$ 10.5 s). A considerable range of prothrombin times were produced by the same daily dose of warfarin. For example, in patients receiving 5 mg of warfarin daily, prothrombin times ranged from 23.5 to 36.5 s (39 $\pm$ 19 s).

A poor correlation (r = 0.390) was obtained between daily dose of warfarin and prothrombin time (Figure 6.2). Similarly, weak correlations were obtained for plasma concentration of racemic warfarin and prothrombin time (r = 0.430) and between plasma concentration of S(-) warfarin and prothrombin time (r = 0.382) (Figures 6.3 and 6.4 respectively).

FIGURE 6.2 Prothrombin time vs. daily dose of warfarin in patients on steady state warfarin therapy involved in the Liverpool study.

Each point (◆) represents an individual patient.
$r = 0.390, P < 0.01$

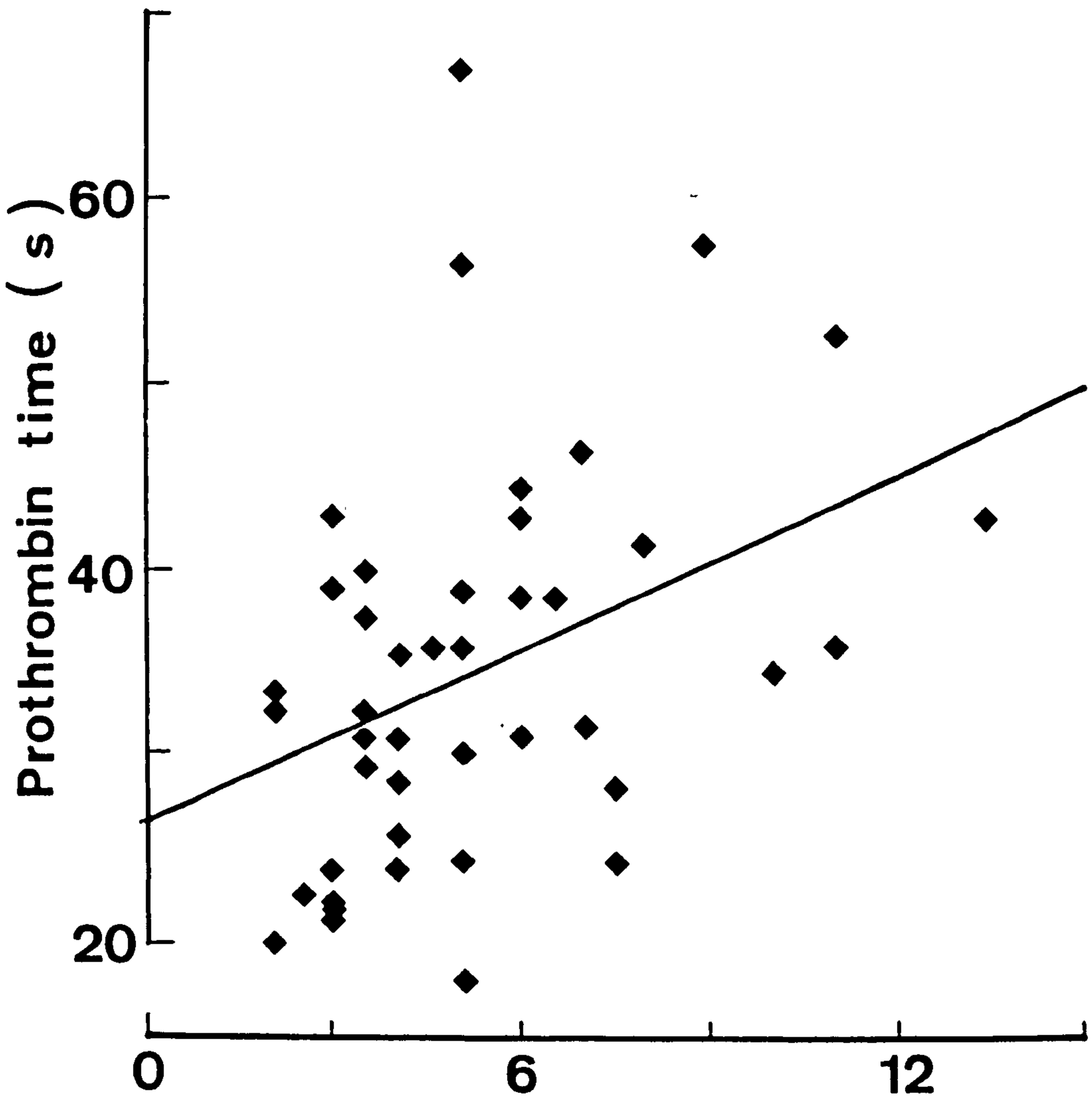


FIGURE 6.3 Prothrombin time vs. plasma concentration of racemic warfarin in patients on steady state warfarin therapy involved in the Liverpool study.

Each point (●) represents an individual patient.

$r = 0.430, P < 0.01$

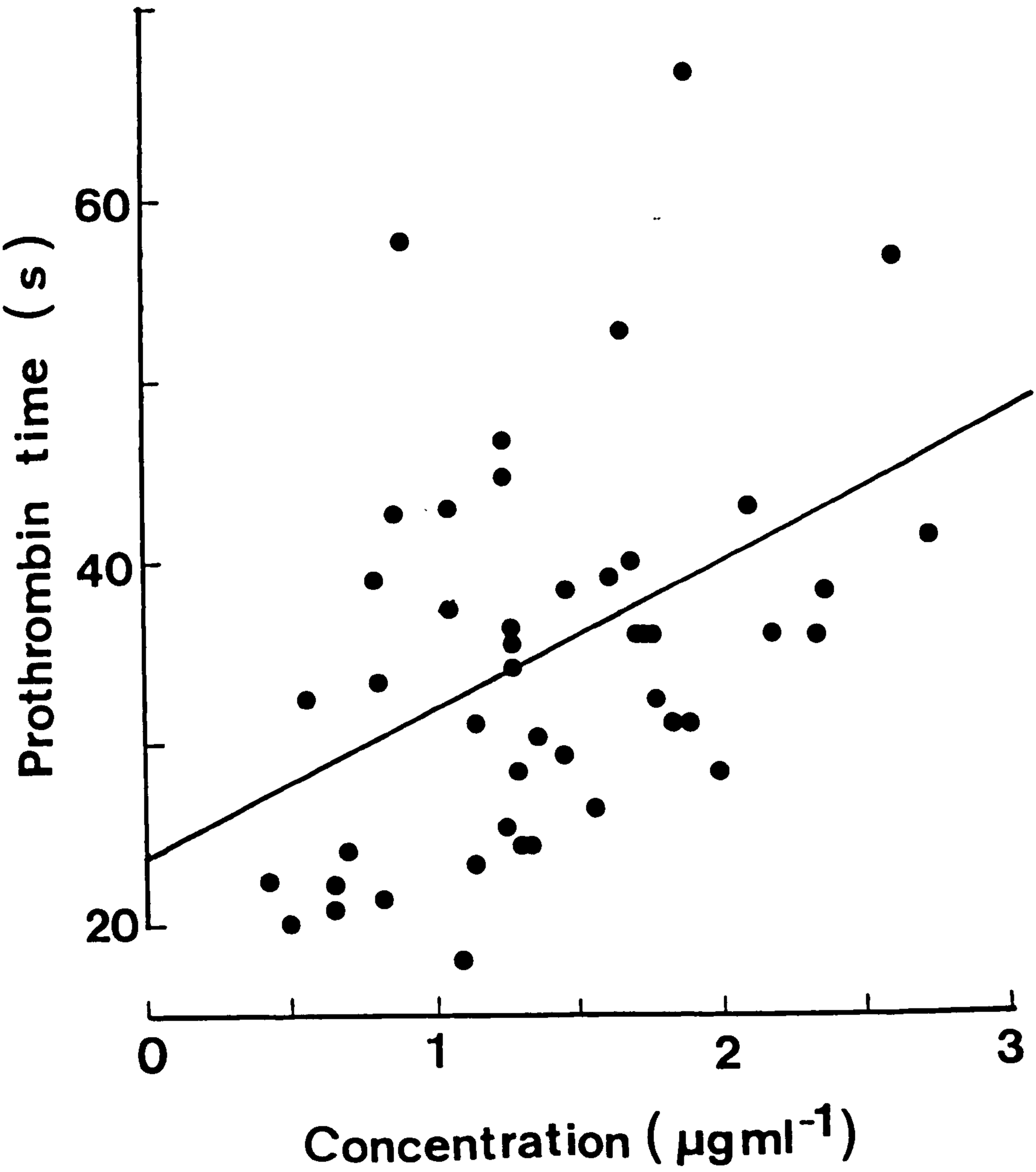
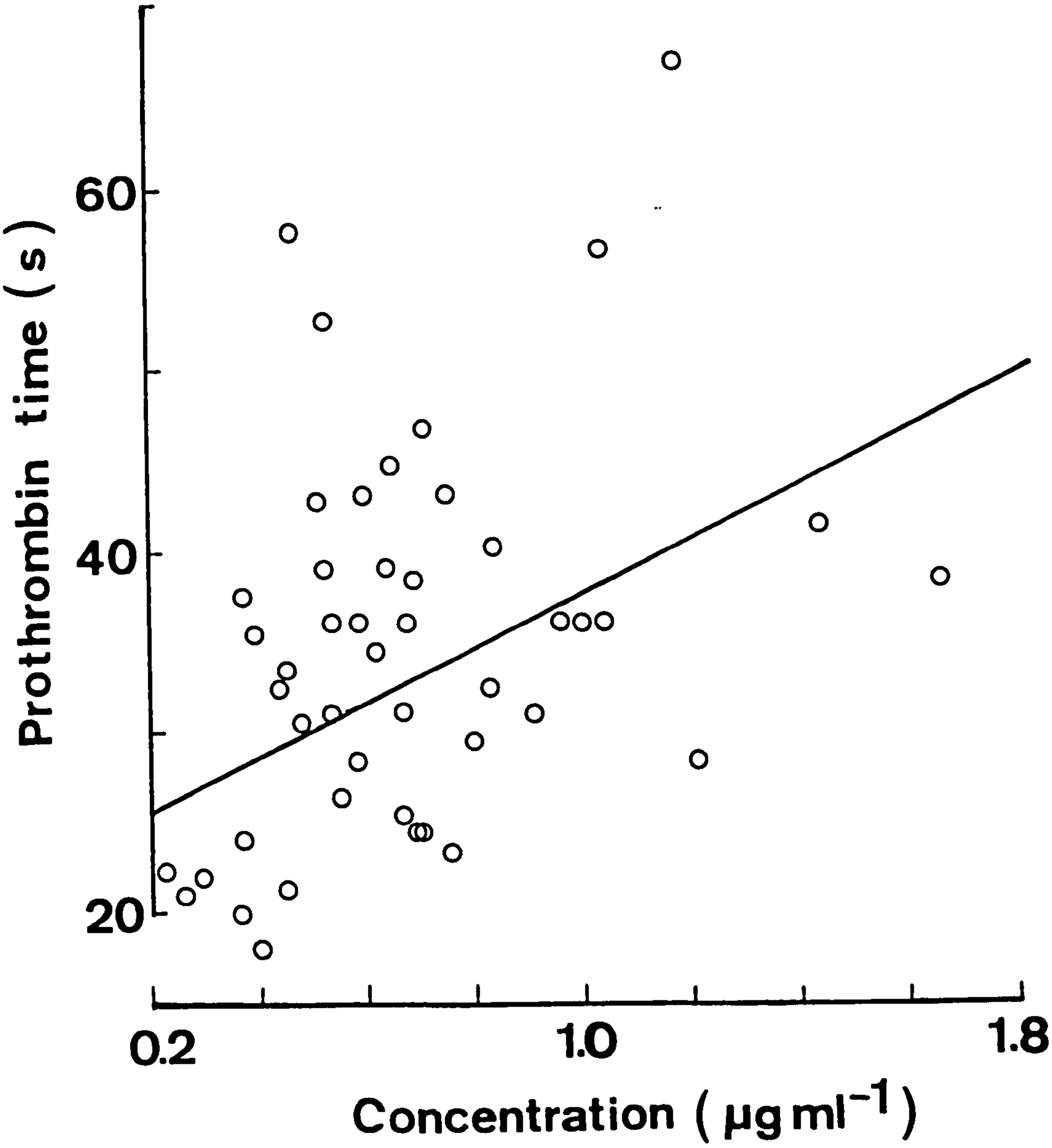


FIGURE 6.4 Prothrombin time vs. plasma concentration of S(-) warfarin in patients on steady state warfarin therapy involved in the Liverpool study.

Each point (O) represents an individual patient.

$r = 0.382, P < 0.05$



MRC Study

In this study daily dose of warfarin ranged from 1.5 to 6.0 mg (3.6 $\pm$ 1.0 mg) and the most commonly prescribed daily dose was 4 mg.

The plasma concentration of racemic warfarin ranged from 0.149 to 1.390 μg ml^{-1} (0.679 $\pm$ 0.263 μg ml^{-1}). There was considerable inter-individual variation in the plasma concentration of racemic warfarin at any given dose. For example, in patients receiving 4 mg of warfarin daily, plasma concentrations ranged from 0.398 to 1.285 μg ml^{-1} (0.750 $\pm$ 0.351 μg ml^{-1}).

Prothrombin time ranged from 12 to 25 s (18 $\pm$ 3 s). In patients receiving 4 mg of warfarin daily, there was a 10 s range in prothrobin time (15 to 25 s).

A poor correlation (r = 0.447) was obtained between daily dose of warfarin and prothrombin time, and an even weaker correlation (r = 0.160) was obtained between plasma warfarin concentration and prothrombin time.

Vitamin K_1 2,3-epoxide in anticoagulated patients

The warfarin requirements of the patients involved in this study ranged from 3 to 11 mg daily (5.2 $\pm$ 2.2 mg daily). From Table 6.2 it can be seen that prothrombin time ranged from 22 to 37 s (26.6 $\pm$ 4.4 s).

The plasma warfarin concentrations determined for the patients involved in this study ranged from 0.51 to 1.37 μg ml^{-1} (0.86 $\pm$ 0.25 μg ml^{-1}; Table 6.1). A two-fold variation (0.51 to 0.95 μg ml^{-1}) in the plasma concentration of warfarin was observed in patients taking 4 mg of warfarin daily.

TABLE 6.1 Clinical details of patients on steady state warfarin

Patient	Age (years)	Dose (mg daily)	Plasma warfarin concentration ($\mu g\ ml^{-1}$)
PR	40	6	0.57
AWO	42	7*	0.70
AWA	44	4	0.51
RA	50	4	0.60
WH	63	4	0.95
AM	64	7	1.37
PE	68	2.5/5*	0.75
JM	66	4*	0.88
ES	64	4*	0.92
AK	63	3*	1.00
DG	63	11	1.19
mean	57	5.2	0.86
± s.d.	10.7	2.2	0.25

* Receiving other medication

A poor correlation (r = 0.295) was obtained between daily dose of warfarin and prothrombin time, and an even weaker correlation (r = 0.038) was obtained between plasma warfarin concentration and prothrombin time.

Following a pharmacological dose of vitamin K_1 (10 mg), all patients had detectable concentrations of vitamin K_1 2,3-epoxide in plasma (Table 6.2). The maximum plasma concentration (Cp_{max}) ranged from 0.286 to 1.186 μg ml^{-1} (0.540 ± 0.252 μg ml^{-1}), 2 to 6 h after administration of vitamin K_1. The area under the curve (AUC) for vitamin K_1 2,3-epoxide ranged from 2.48 to 18.86 μg ml^{-1} h (8.50 ± 4.69 μg ml^{-1} h).

A lack of correlation was demonstrated between AUC for vitamin K_1 2,3-epoxide and prothrombin time (r = 0.182) and also between Cp_{max} for vitamin K_1 2,3-epoxide and prothrombin time (r = -0.240) (Figures 6.5 and 6.6 respectively).

TABLE 6.2 Pharmacokinetics of vitamin K_1 2,3-epoxide and prothrombin time in patients on steady state warfarin.

Patient	Prothrombin time (s)	Vitamin K_1 2,3-epoxide AUC ($\mu g\ ml^{-1}\ h$)	Vitamin K_1 2,3-epoxide Cp_{max} ($\mu g\ ml^{-1}$)
PR	24	2.48	0.413
AWO	32	18.86	0.394
AWA	26	10.33	1.186
RA	24.5	6.44	0.444
WH	25.5	10.66	0.549
AM	25	10.68	0.472
PE	29	7.41	0.590
JM	22	2.88	0.293
ES	25	10.60	0.580
AK	37	3.86	0.286
DG	23	9.28	0.729
mean	26.6	8.50	0.540
± s.d.	4.4	4.69	0.252

FIGURE 6.5 Prothrombin time vs. area under the concentration-time curve for vitamin K_1 2,3-epoxide (AUC KO) in patients on steady state warfarin therapy following intravenous administration of vitamin K_1 (10mg).

Each point (●) represents an individual patient.
r = 0.182.

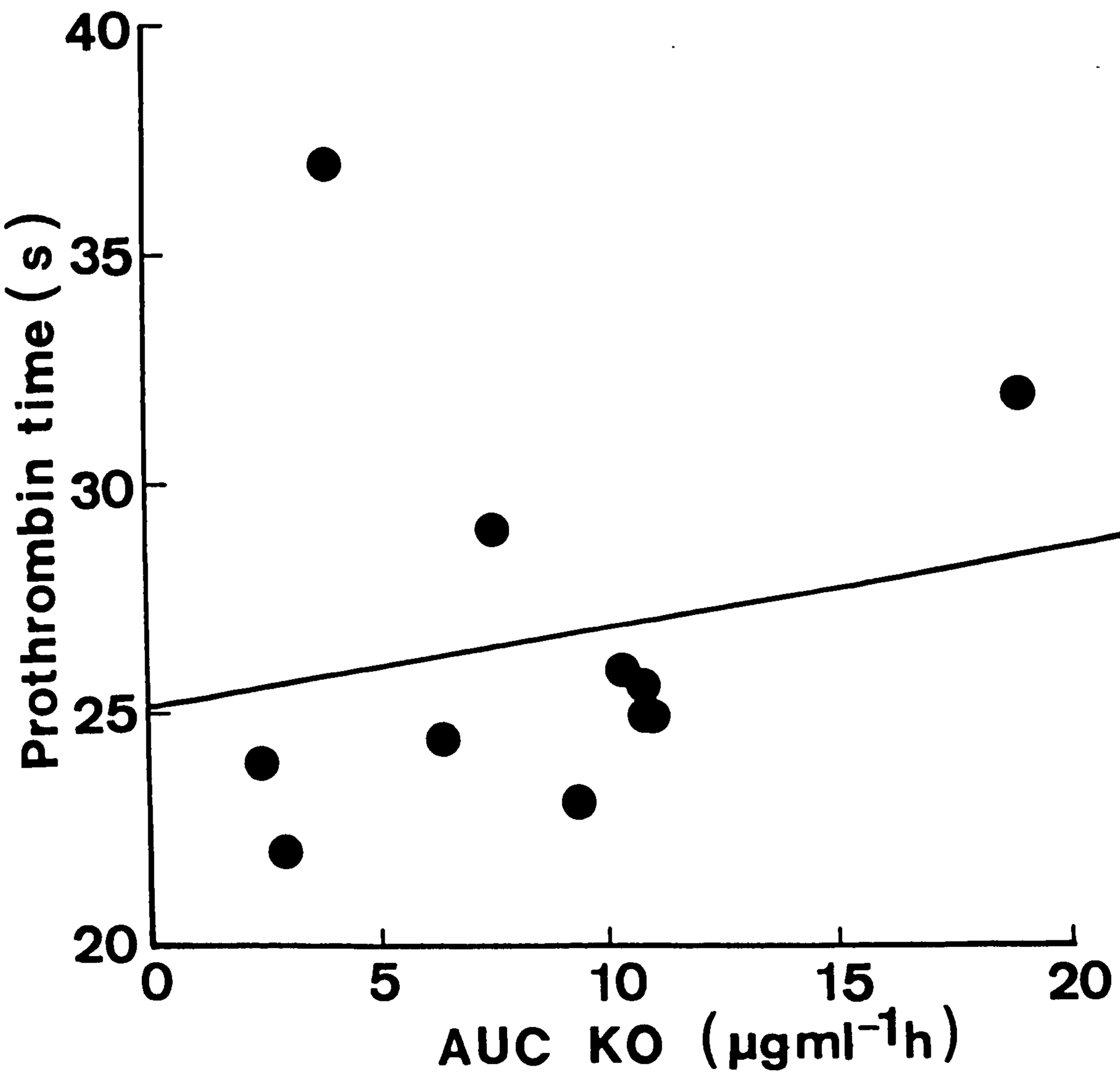
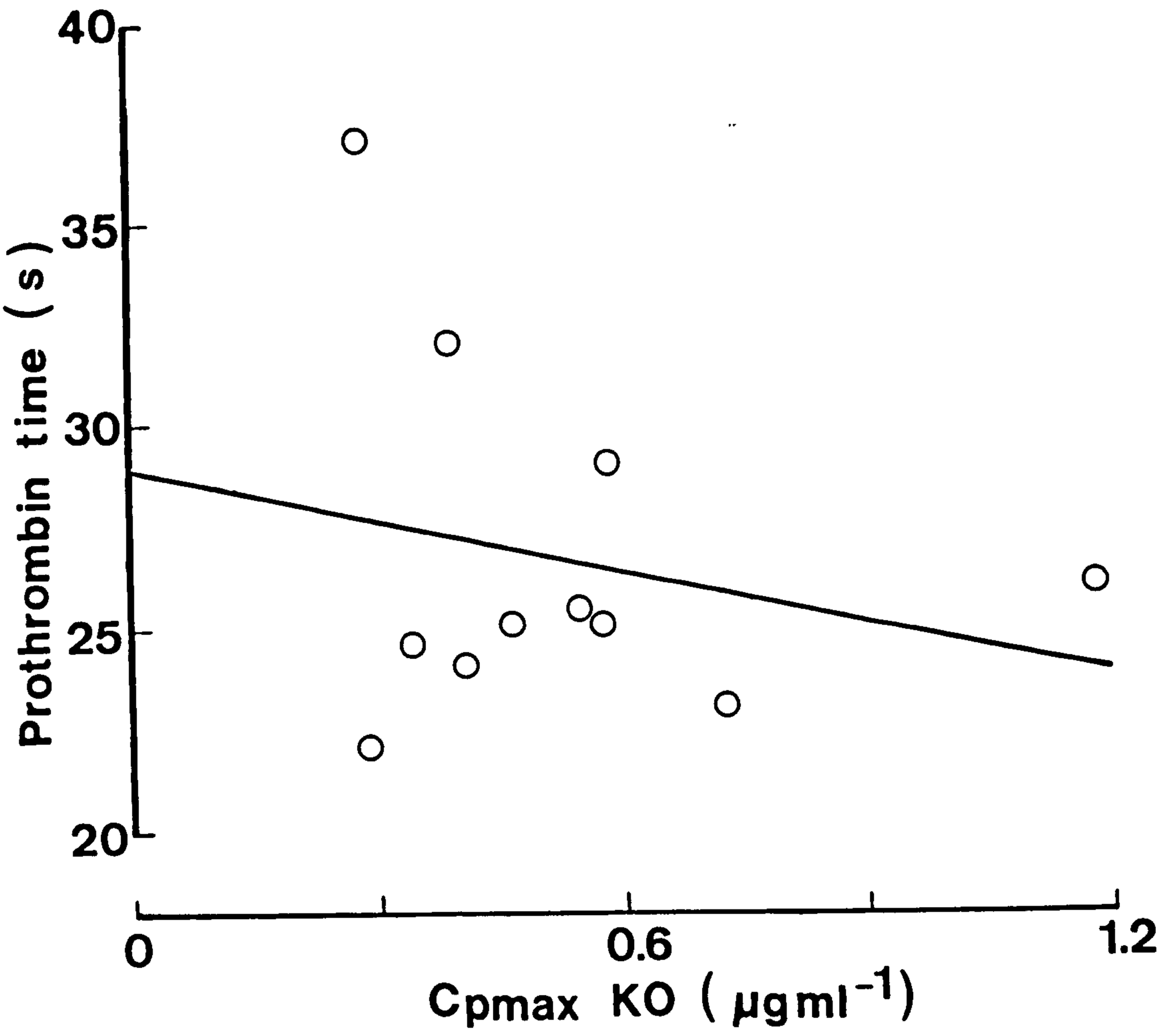


FIGURE 6.6 Prothrombin tims vs. maximum plasma concentration of vitamin K_1 2,3-epoxide (Cp_{max} KO) in patients on steady state warfarin therapy following intravenous administration of vitamin K_1 (10 mg).

Each point (O) represents an individual patient.
r = -0.240.



6.4 Discussion

Many of the studies in which the pharmacokinetics and the pharmacodynamics of warfarin and its enantiomers have been investigated, have involved single doses of warfarin in young, healthy volunteers (Breckenridge et al., 1974; Wingard et al., 1978; Hignite et al., 1980). However, to relate to the clinical situation of anticoagulant therapy, it is important, whenever possible to perform studies in groups of patients on steady state warfarin therapy.

The studies described in this chapter allowed the relationships between daily dose of warfarin, the pharmacodynamic effect and biochemical effect of warfarin, to be investigated in patients with a view to establishing a relationship which could be employed to more accurately predict the pharmacological effect of warfarin.

The patients involved in the Liverpool study and the vitamin K_1 2,3-epoxide in anticoagulated patients study displayed dose requirements of warfarin, plasma warfarin concentrations and prothrombin times similar to each other and similar to those reported by other investigators (Yacobi et al., 1976; Bentley et al., 1986). In contrast, the patients involved in the MRC study were taking lower daily doses of warfarin as prophylactic therapy and as a consequence displayed lower plasma warfarin concentrations and prothrombin times.

The results from the three studies described in this chapter suggest that measurement of plasma warfarin concentration is no better than daily dose of warfarin as a predictor of pharmacological response as determined by prothrombin time. Furthermore, the predictive parameter r^2 was calculated to be 0.146 for plasma S(-) war-

farin concentration against prothrombin time, illustrating that plasma concentration of S(-) warfarin is also a poor predictor of anticoagulant effect, even though a recent theoretical model has confirmed the greater potency of S(-) warfarin in man and has suggested that the pharmacological effect following racemic warfarin resides exclusively with the S enantiomer (Chan et al., 1986).

In the final study described in this chapter, vitamin K_1 2,3-epoxide was detected in the plasma of all the patients involved, 2 to 6 h after the administration of vitamin K_1. A ten-fold inter-patient variation was observed for both AUC and Cp_{max} for vitamin K_1 2,3-epoxide. This is probably due to the inter-individual variation in both the degree of inhibition of the enzyme vitamin K epoxide reductase and the rate of elimination of the epoxide.

The presence of vitamin K_1 2,3-epoxide in the plasma of individuals on anticoagulant therapy following a dose of vitamin K_1, is consistent with previous findings in both healthy volunteers (Park et al., 1984) and the rabbit (Hart et al., 1984). Thus vitamin K_1 2,3-epoxide provides a marker of coumarin action in addition to prothrombin time. However, neither AUC or Cp_{max} for vitamin K_1 2,3-epoxide were useful parameters for the prediction of prothrombin time, since the predictive value r^2 was determined to be 0.033 for AUC and 0.058 for Cp_{max} for vitamin K_1 2,3-epoxide. The latter showing a negative correlation.

6.5 Conclusions

The aim of the studies described in this chapter was to determine the relationships between daily dose of warfarin, the plasma concentrations of racemic and S(-) warfarin, the biochemical effect of warfarin, as determined by plasma concentration of vitamin K_1 2,3-epoxide and prothrombin time in patients on steady state warfarin therapy, with a view to accurate prediction of the pharmacological effect of warfarin.

The results of all three studies indicated that plasma warfarin concentration is no better as a predictor of pharmacological effect than daily dose of warfarin. This suggests that inter-individual differences in receptor sensitivity are an important factor in determining inter-individual variation in pharmacological response.

Plasma concentrations of vitamin K_1 2,3-epoxide after intravenous administration of a pharmacological dose of vitamin K_1 were therefore investigated as a marker of warfarin action. However, the correlations obtained between vitamin K_1 2,3-epoxide and prothrombin time were insufficient in this small number of subjects, to propose this biochemical marker as a predictor of the pharmacological effect of warfarin. Thus the apparent variability in receptor sensitivity to warfarin may reflect inter-individual differences in protein (clotting factor) metabolism rather than vitamin K_1 metabolism.

CHAPTER 7

INVESTIGATION OF THE PHARMACOLOGY OF LOW DAILY DOSES OF WARFARIN AND THE ENANTIOMERS OF WARFARIN IN VOLUNTEERS

7.1 Introduction

To further our understanding of the pharmacology of warfarin it is important whenever possible to undertake patient studies rather than volunteer studies.

In Chapter 6, three clinical studies were described in which the pharmacological and the biochemical effects of warfarin were related to its pharmacokinetics in patients on steady state warfarin therapy. However certain questions arise from the clinical situation which must be answered by undertaking volunteer studies. This type of investigation allows better control of the experiment, thus enabling a specific matter to be investigated with greater precision.

In Chapter 6 inhibition of the enzyme vitamin K epoxide reductase by warfarin was seen to produce measurable concentrations of vitamin K_1 2,3-epoxide in patients on steady state warfarin therapy. This finding is consistent with previous results in man (Park et al., 1984) and experimental animals (Caldwell et al., 1974; Park et al., 1979) and advocates vitamin K_1 2,3-epoxide as a marker of 4-hydroxycoumarin action.

Clinicians around the world have reawakened their interest in the use of anticoagulants as it has been suggested that less intense (low dose) therapy may be used for the treatment of thrombo-embolic disorders (Francis et al., 1983).

The first study described in this chapter was carried out in volunteers to investigate the relationships between the pharmacokinetics, the pharmacodynamics and the biochemical effect of warfarin during anticoagulation with low daily doses of warfarin. In addition it provides an opportunity to evaluate the sensitivity of prothrombin time

and plasma concentration of vitamin K_1 2,3-epoxide at doses of warfarin lower than those conventionally employed.

When administered clinically, warfarin consists of a racemic mixture of two optically active isomers. Many studies have investigated the pharmacokinetics and the pharmacodynamics of these enantiomers in man and S(-) warfarin has been found to be more potent despite having a shorter half-life than R(+) warfarin (Breckenridge et al., 1974; O'Reilly, 1974a; Wingard et al., 1978).

Previous work involving the administration of single doses of the enantiomers of warfarin to man have resulted in similar plasma concentrations of vitamin K_1 2,3-epoxide following a dose of vitamin K_1 (Shearer et al., 1977; Choonara et al., 1986). However, studies in rats have shown a stereoselective difference in 4-hydroxycoumarin-induced vitamin K_1 2,3-epoxide formation (Schmidt et al., 1979; Bell and Ren, 1981). A possible explanation for the lack of stereoselective effect in man is that large doses of warfarin were used in previous studies, and as a consequence, the enzyme vitamin K epoxide reductase was sufficiently inhibited by both enantiomers to produce a maximum amount of vitamin K_1 2,3-epoxide.

The second study in this chapter involves steady state plasma concentrations of the enantiomers of warfarin obtained using a low dose of warfarin with a view to ascertaining whether a stereoselective effect exists in man, and to determine its relationship with the anticoagulant potency of the individual enantiomers.

7.2 Plan of Study

Investigation of the Pharmacology of Low Daily Doses of Warfarin in Volunteers

Seven healthy male volunteers took either (i) 1 mg or, (ii) 0.2 mg of racemic warfarin daily for 2 weeks in a randomised order. There was a minimum rest period of 4 weeks between the two parts of the study. Each individual gave informed consent and approval was obtained from the Mersey Regional Ethics Committee.

12, 13, 14, 15 and 16 days after initiation of warfarin therapy, venous blood samples (10 ml) were collected into heparinised blood tubes 24 h after daily warfarin administration. Steady state plasma warfarin concentrations were determined as described in Section 2.3.1. 12, 13 and 14 days after the initiation of warfarin therapy, venous blood samples (0.9 ml) were collected for the immediate determination of prothrombin time as was described in section 2.3.7.

Once steady state plasma warfarin concentrations had been reached (2 weeks), each volunteer received a single intravenous dose of vitamin K_1 (10 mg) diluted in 0.9% saline (10 ml) and given over a period of 10 min. They continued to receive warfarin for 96 h after the administration of vitamin K_1. Venous blood samples (10 ml) were collected into heparinised blood tubes prior to warfarin administration and at 0, 0.5, 1, 2, 4, 6, 8, 24, 48, 72 and 96 h after vitamin K_1 for the determination of plasma vitamin K_1 2,3-epoxide concentrations as described in section 2.3.3.

Commercially available tablets were used for the 1 mg dose of warfarin, and gelatin capsules containing 0.2 mg of warfarin were prepared by the addition of lactose prior to packing.

Results are expressed as mean ± standard deviation of mean (mean ± s.d.). Levels of significance were determined using the paired Student's t test.

Investigation of the Pharmacology of the Enantiomers of Warfarin in Volunteers

Five healthy male volunteers took 1 mg of warfarin (R(+) warfarin or S(-) warfarin) daily for 2 weeks in a randomised order. There was a minimum rest period of 4 weeks between the two parts of the study. Each individual gave informed consent and approval was obtained from the Mersey Regional Ethics Committee.

12, 13, 14, 15 and 16 days after initiation of warfarin therapy, venous blood samples (10 ml) were collected into heparinised blood tubes 24 h after daily warfarin administration. Steady state plasma warfarin concentrations were determined as described in Section 2.3.1. 12, 13 and 14 days after the initiation of warfarin therapy, venous blood samples (0.9 ml) were collected into trisodium citrate solution and prothrombin time was determined as described in section 2.3.7.

Once steady state plasma warfarin concentrations had been reached (2 weeks), each volunteer received a single intravenous dose of vitamin K_1 (10 mg) diluted in 0.9% saline (10 ml) and given over a period of 10 min. They continued to take 1 mg of warfarin daily for 72 h after the administration of vitamin K_1. Venous blood samples (10 ml) were collected into heparinised blood tubes prior to warfarin administration and at 0, 0.5, 1, 2, 4, 6, 8, 12, 24, 48 and 72 h after vitamin K_1 for the determination of plasma vitamin K_1 2,3-epoxide concentrations as described in section 2.3.3.

The warfarin capsules were prepared by the addition of lactose to the warfarin prior to packing in gelatin capsules.

The results are expressed as mean ± standard deviation of mean (mean ± s.d.). Levels of significance were determined using the paired Student's t test.

7.3 Results

Investigation of the Pharmacology of Low Daily Doses of Warfarin in Volunteers

It can be seen from Table 7.1 that the plasma concentrations of warfarin were significantly ($P < 0.001$) elevated after the administration of 1 mg daily when compared with 0.2 mg daily. Following 0.2 mg of warfarin daily, the plasma concentration of warfarin was below the limit of sensitivity of the assay (< 50 ng ml^{-1}) in one individual (PW). This illustrates the more rapid plasma clearance of warfarin in this individual assuming that bioavailability is 100%.

The pharmacodynamic effect of warfarin is illustrated in Table 7.2. The higher dose of warfarin caused a small but significant ($P < 0.05$) increase in the mean prothrombin time (0.9 s). However, following the lower dose of warfarin very little, if any, effect on prothrombin time was seen in the volunteers.

All seven individuals had detectable plasma concentrations of vitamin K_1 2,3-epoxide after both doses of warfarin. Both the maximum plasma concentration of vitamin K_1 2,3-epoxide (Cp_{max}) and the area under the curve (AUC) for the epoxide were significantly ($P < 0.01$) higher after 1 mg of warfarin daily than after 0.2 mg daily (Table 7.2).

Investigation of the Pharmacology of the Enantiomers of Warfarin in Volunteers

Table 7.3 illustrates the plasma clearance values obtained for both R(+) warfarin and S(-) warfarin in the five individuals involved in the present study. It can be seen that there was a significantly ($P < 0.05$) greater plasma clearance of S(-) warfarin when compared to

TABLE 7.1 Plasma concentrations of warfarin at steady state in volunteers

Volunteer	Plasma warfarin concentration (ng ml^{-1})	
	1 mg daily	0.2 mg daily
IC	269	99
PW	180	ND
BH	157	72
MT	242	92
GP	215	70
JB	260	41
JF	292	47
Mean	231*	60
± s.d.	49	34

ND - non detectable

* - Significantly different from 0.2 mg daily, $P < 0.001$

TABLE 7.2 Prothrombin times and the pharmacokinetics of vitamin K_1 2,3-epoxide in volunteers on steady state warfarin.

	Prothrombin time (s)		
	Initial	On Warfarin	Increase
1 mg mean	13.0	13.9	0.9
± s.d.	0.3	0.9	0.8
0.2 mg mean	13.0	13.0	0.2
± s.d.	0.4	0.5	0.2

	Vitamin K_1 2,3-epoxide	
	Cp_{max} (ng ml^{-1})	AUC (ng ml^{-1} h)
1 mg mean	271	1563
± s.d.	84	411
0.2 mg mean	94	442
± s.d.	59	353

Cp_{max} - maximum plasma concentration

AUC - area under the curve

TABLE 7.3 The pharmacokinetics of warfarin and the pharmacodynamic response to warfarin in volunteers on steady state warfarin.

Volunteer		Plasma warfarin concentration (ng ml^{-1})	Warfarin clearance (ml h^{-1} kg^{-1})	Increase in prothrombin time (s)
D A	R	314	1.33	1.5
	S	175	2.38	2.9
R C	R	196	2.66	0.6
	S	103	5.06	1.0
N K	R	220	2.56	0.7
	S	188	3.00	1.4
P R	R	277	2.35	1.0
	S	176	3.70	1.1
D T	R	235	2.16	1.0
	S	235	2.16	2.5
mean ± s.d.	R	248	2.21	0.96
mean ± s.d.	S	175*	3.26*	1.78*

R - R(+) Warfarin

S - S(-) Warfarin

* Significantly different from R, $P < 0.05$

R(+) warfarin. As a consequence the trough steady state plasma concentrations of S(-) warfarin were significantly ($P < 0.05$) lower than those of R(+) warfarin.

There was a significantly ($P < 0.05$) greater increase in prothrombin time after the administration of S(-) warfarin than after R(+) warfarin (Table 7.3). The prothrombin time returned to within 0.2s of its initial value (11.5 to 13.4 s) in all five volunteers after the administration of vitamin K_1. The coefficient of variation for the determination of prothrombin time was 1.2%.

Increase in prothrombin time against the log of the plasma warfarin concentration produced concentration-response curves for the enantiomers of warfarin (Figure 7.1). That for S(-) warfarin can be seen to lie to the left of that for the R enantiomer, illustrating the greater potency of S(-) warfarin.

Following the administration of a pharmacological dose of vitamin K_1 (10 mg) both the maximum plasma concentrations (Cp_{max}) and the area under the curve (AUC) for vitamin K_1 2,3-epoxide were significantly ($P < 0.05$) higher after S(-) warfarin than after R(+) warfarin (Table 7.4).

FIGURE 7.1 Increase in prothrombin time vs. log plasma concentration of warfarin in volunteers on steady state R(+) warfarin (■) and S(-) warfarin (★) therapy (1 mg daily).

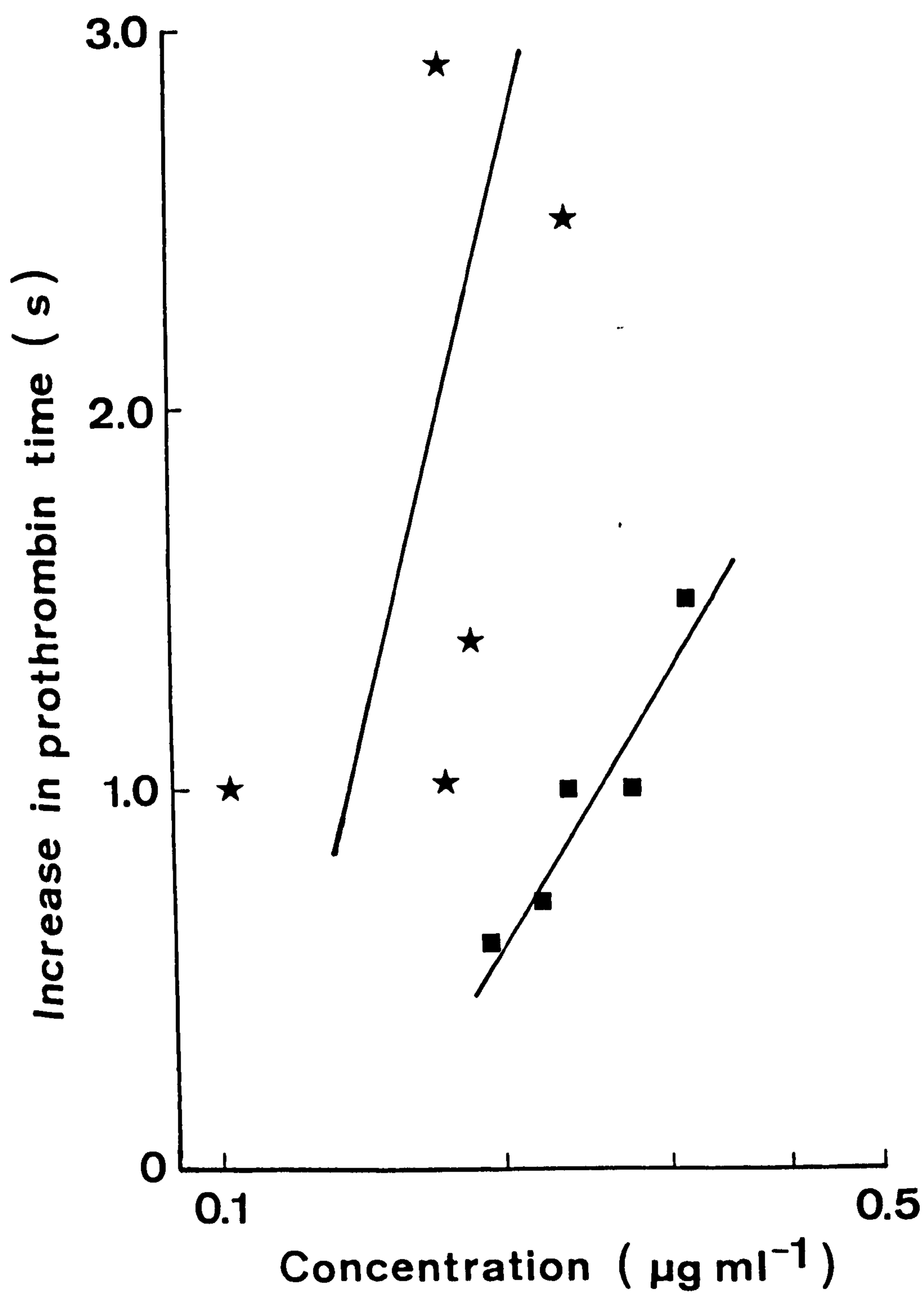


TABLE 7.4 The pharmacokinetics of vitamin K_1 2,3-epoxide in volunteers on steady state warfarin.

Volunteer		Vitamin K_1 2,3-epoxide	
		Cp_{max} (ng ml^{-1})	AUC (ng ml^{-1} h)
D A	R	135	703
	S	149	900
R C	R	132	784
	S	256	1470
N K	R	111	549
	S	205	917
P R	R	114	747
	S	218	1458
D T	R	186	1015
	S	202	1140
mean ± s.d.	R	136	760
mean ± s.d.	S	206*	1177*

R - R(+) Warfarin Cp_{max} - maximum plasma concentration

S - S(-) Warfarin AUC - area under the curve.

* Significantly different from R, $P < 0.05$.

7.4 Discussion

Investigation of the Pharmacology of Low Daily Doses of Warfarin in Volunteers

The doses of warfarin employed in the present study are much lower than those conventionally administered in the clinical situation. However it has recently been advocated that low dose warfarin therapy is suitable for the treatment of thrombo-embolic disorders (Francis et al., 1983).

A two-fold inter-individual variation in plasma warfarin concentrations was obtained with both doses of warfarin. This is similar to the variation obtained in patients on 4 mg of warfarin daily as described in Chapter 6.

The detection of vitamin K_1 2,3-epoxide in all seven individuals after both 1 mg and 0.2 mg of warfarin daily, is consistent with previous findings in patients on steady state warfarin therapy (Chapter 6) and provides further evidence for the inhibition of the enzyme vitamin K epoxide reductase by warfarin (Bell and Matschiner, 1972; Whitlon et al., 1978).

The results of the present study suggest that there is a dose-dependent inhibition of vitamin K epoxide reductase. In terms of both Cp_{max} and AUC, the plasma concentrations of vitamin K_1 2,3-epoxide were significantly higher after 1 mg of warfarin daily than after 0.2 mg daily.

Using data generated in the present study and that described in Chapter 6, the dose/concentration-response curves illustrated in Figures 7.2 and 7.3 were obtained. Figure 7.2 illustrates the range of AUC values obtained for vitamin K_1 2,3-epoxide at different daily

FIGURE 7.2 Area under the plasma concentration-time curve for vitamin K_1 2,3-epoxide vs log daily dose of warfarin in patients and volunteers on steady state warfarin therapy following intravenous administration of vitamin K_1 (10mg). Results are expressed as means and vertical bars show s.d. mean.

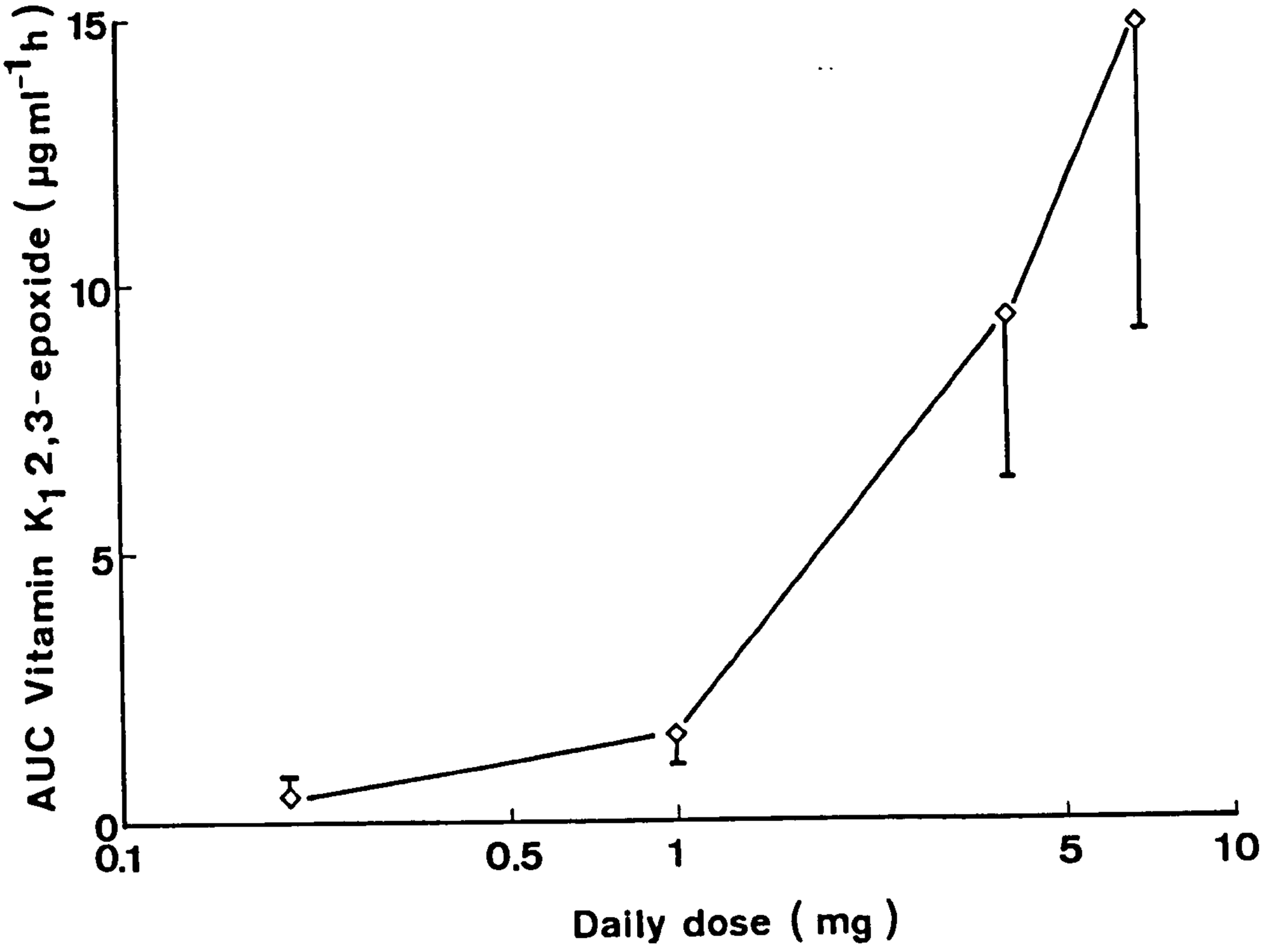
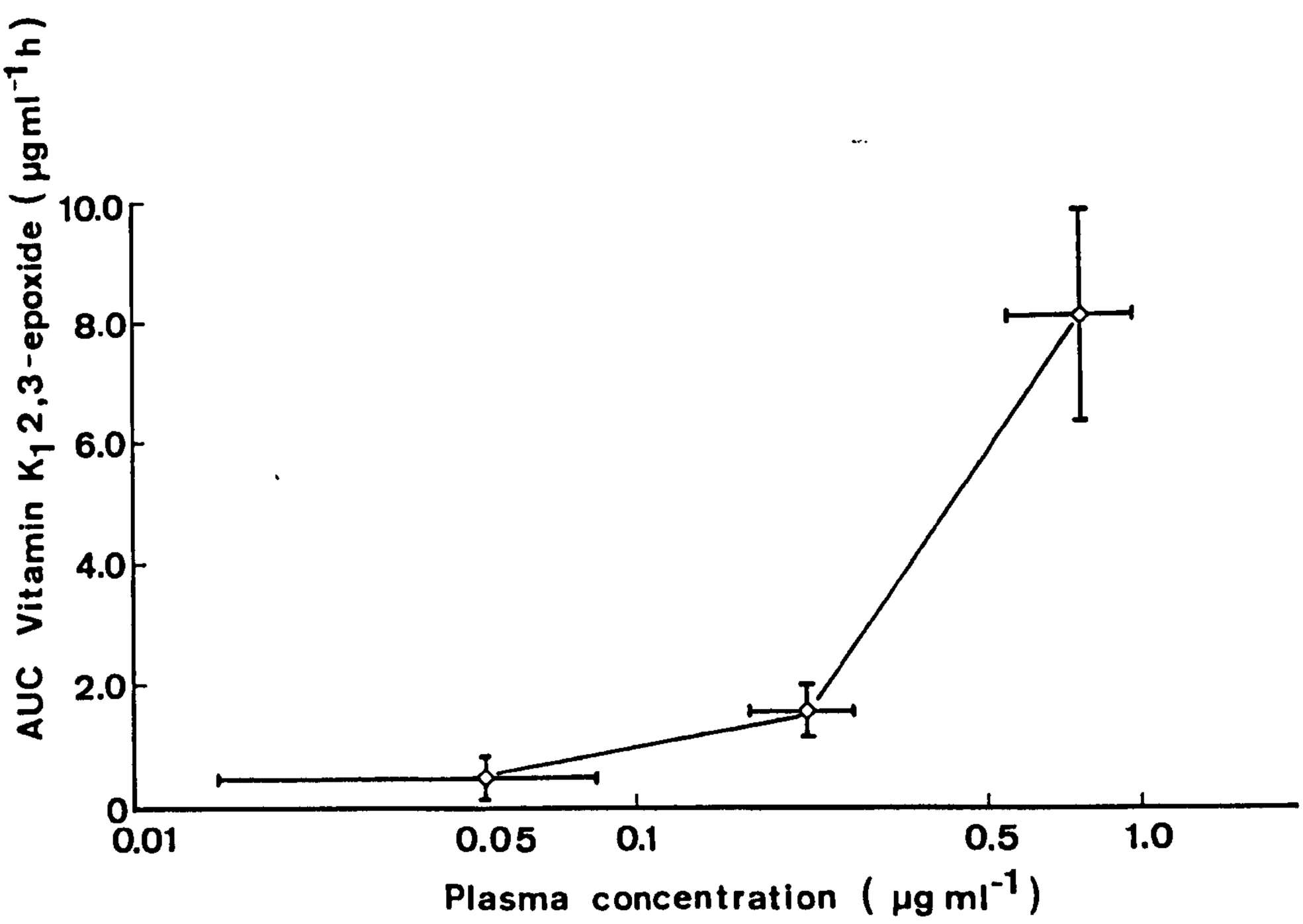


FIGURE 7.3 Area under the plasma concentration-time curve for vitamin K_1 2,3-epoxide vs. log plasma concentration of warfarin in patients and volunteers on steady state warfarin therapy following intravenous administration of vitamin K_1 (10mg).

Results are expressed as means and the vertical and horizontal bars show s.d. means.



doses of warfarin. 0.2 mg of warfarin daily, produced a ten-fold inter-individual variation in AUC for vitamin K_1 2,3-epoxide, whereas 1 mg produced only a two-fold variation in this parameter. The variation at all doses reflects the inter-individual variation in both the pharmacokinetics and the pharmacodynamics of warfarin. Nevertheless the mean data clearly shows a dose-dependent effect of warfarin on the accumulation of vitamin K_1 2,3-epoxide. Figure 7.3 illustrates AUC for vitamin K_1 2,3-epoxide plotted against the log of plasma warfarin. Although there are only three points, the results suggest a concentration-dependent inhibition of vitamin K epoxide reductase as measured by the biochemical marker, vitamin K_1 2,3-epoxide. This effect has previously been investigated resulting in the production of a dose-response curve illustrating the relationship between a single dose of warfarin and Cp_{max} for vitamin K_1 2,3-epoxide 1 to 4h after the administration of a tracer dose of vitamin K_1 to healthy volunteers (Shearer et al., 1977). Thus dose-dependent inhibition of vitamin K_1 epoxide reductase may be observed following either a tracer, or a pharmacological dose of vitamin K_1 during anticoagulation with either a single dose or steady state warfarin.

The pharmacodynamic effect of warfarin was monitored by the measurement of prothrombin time. The mean data shows a small, but significant, increase in prothrombin time following 1 mg of warfarin daily when compared to 0.2 mg daily. There was no measurable pharmacodynamic effect following 0.2 mg of warfarin daily. This suggests that the detection of plasma vitamin K_1 2,3-epoxide is a more sensitive indicator of the presence of 4-hydroxycoumarin anticoagulants than prothrombin time at low doses of warfarin. Using data obtained in the present study and that obtained in Chapter 6, increase in pro-

thrombin time against the log of the dose of warfarin (Figure 7.4) and increase in prothrombin time against the log of the plasma concentration of warfarin (Figure 7.5) suggest a direct relationship between both dose and plasma concentration of warfarin and pharmacodynamic effect.

The results of the present study appear to raise important questions about the mode of action of warfarin. It is evident that administration of 0.2 mg of warfarin daily to healthy volunteers results in the inhibition of vitamin K epoxide reductase as determined by plasma concentration of vitamin K_1 2,3-epoxide, without any change in clotting factor activity as determined by a change in prothrombin time. It seems likely that the cause of this differential effect is due to the relative insensitivity of the prothrombin time assay as a measure of clotting factor synthesis. Recently it has become possible to measure γ-carboxylated prothrombin (Blanchard et al., 1983) and this appears to be a much more sensitive marker of the functional aspects of coagulation than prothrombin time. In a study in which the presence of vitamin K_1 deficiency in chronic gastro-intestinal disorders was investigated, the measurement of abnormal prothrombin deficient in γ-carboxyglutamic acid was shown to be approximately 1000-fold more sensitive than prothrombin time for the diagnosis of vitamin K_1 deficiency (Krasinski et al., 1985). It would therefore be of interest to measure the relationship between plasma warfarin concentrations, vitamin K_1 2,3-epoxide accumulation and prothrombin antigen by radioimmunoassay.

An additional explanation of the differential effect observed in the present study is that like many other biological systems, the enzyme vitamin K epoxide reductase may have a threshold of inhibition

FIGURE 7.4 Increase in prothrombin time from control value vs. log daily dose of warfarin in patients and volunteers on steady state warfarin therapy.

Results are expressed as means and vertical bars show s.d. mean.

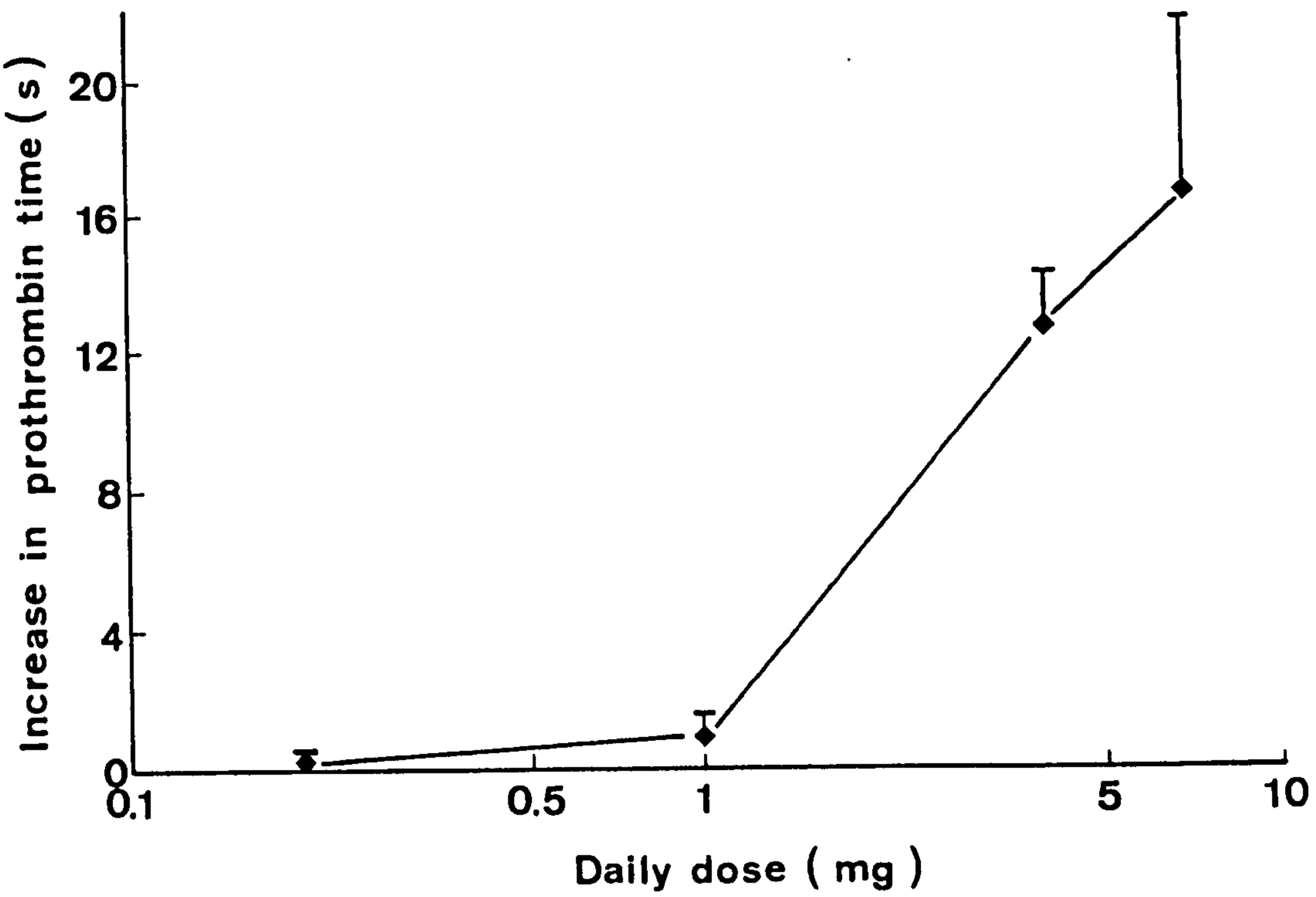
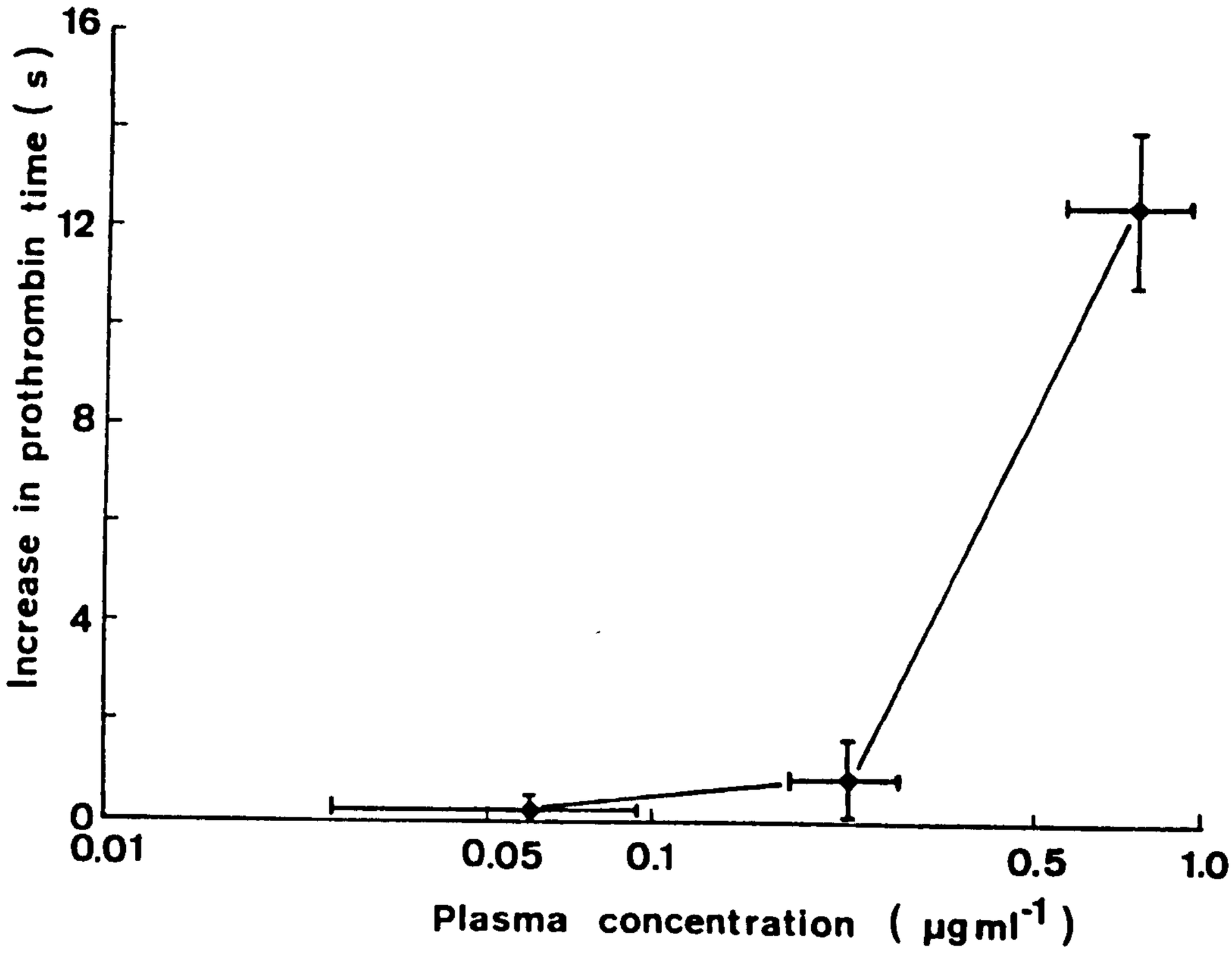


FIGURE 7.5 Increase in prothrombin time from control vs. log plasma concentration of warfarin in patients and volunteers on steady state warfarin therapy.

Results are expressed as means and vertical and horizontal bars show s.d. means.



with respect to gross pharmacological effect. For example. the enzyme carbonic anhydrase at its site of action in the renal cortex, requires more than 99% inhibition before a diuretic effect becomes apparent (Mudge, 1980). Likewise, the enzyme monoamine oxidase present in the type B form in human platelets, requires greater than 85% inhibition before an antidepressant effect is observed (Robinson et al., 1978). Thus it seems possible that below a certain degree of inhibition there may be an elevation of vitamin K_1 2,3-epoxide concentrations. but no change in clotting factor activity, or even prothrombin antigen concentration.

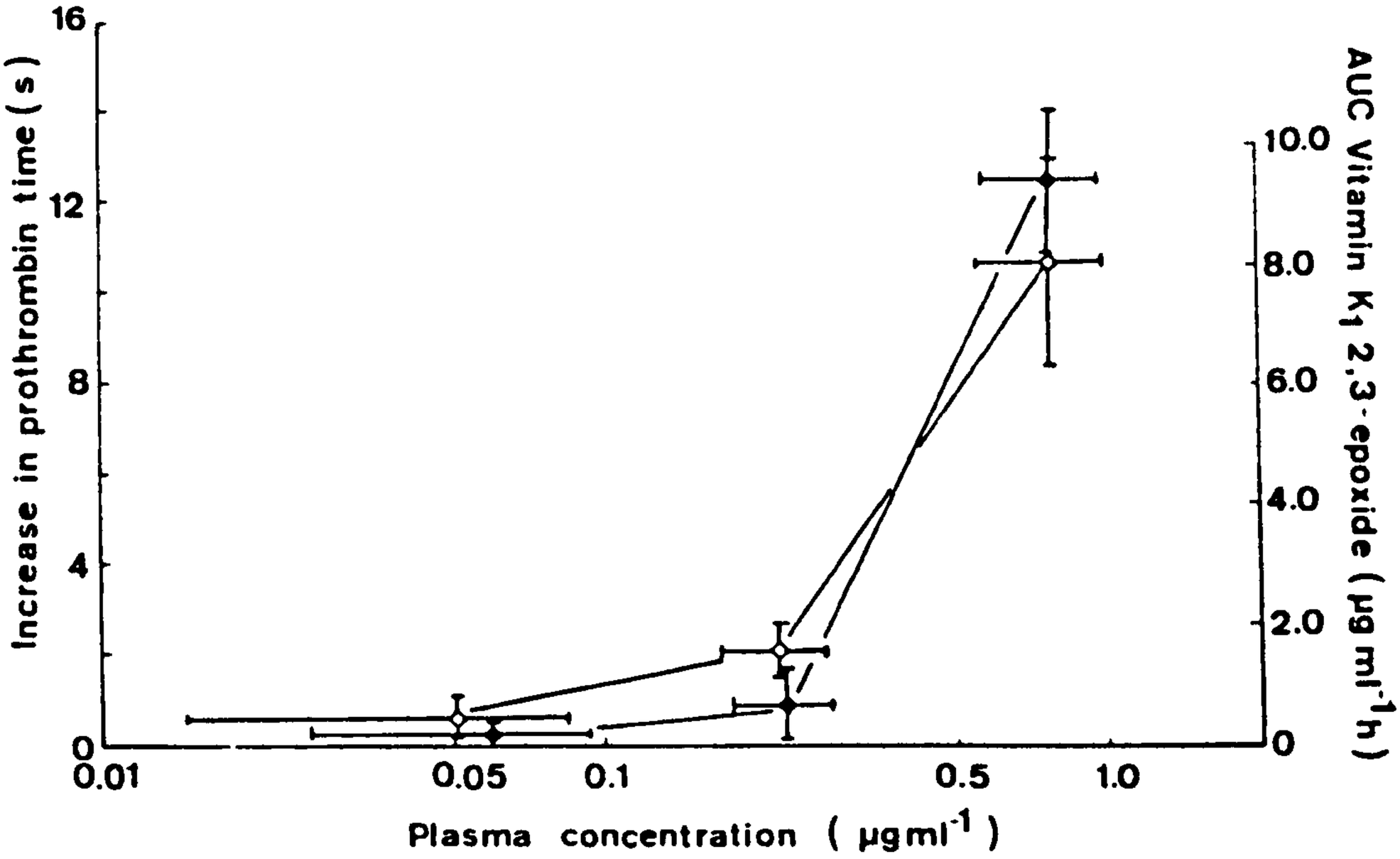
However, if the concentration-response curves obtained for concentration of warfarin against increase in prothrombin time and concentration of warfarin against AUC for vitamin K_1 2,3-epoxide are compared (Figure 7.6), it can be seen that they are similar to each other. Thus the differential effect observed in the present study is due to the fact that the prothrombin time assay is a less sensitive marker of the effects of low doses of warfarin than is plasma concentration of vitamin K_1 2,3-epoxide.

Investigation of the Pharmacology of the Enantiomers of Warfarin in Volunteers

Several studies have reported that S(-) warfarin is more potent than R(+) warfarin in man. The mean potency of S(-) warfarin has been calculated to be 3.8 (Breckenridge et al., 1974), 3.4 (O'Reilly. 1974a) and 2.7 (Wingard et al., 1978) times that of R(+) warfarin. In these studies a significantly greater plasma half-life of R(+) warfarin was observed when compared to that of S(-) warfarin. Consistent with this observation, the plasma clearance of the S enantiomer was found to

FIGURE 7.6 Concentration-response curves for increase in prothrombin time vs. plasma concentration of warfarin and area under the concentration-time curve for vitamin K_1 2,3-epoxide vs. plasma concentration of warfarin in patients and volunteers on steady state warfarin therapy.

Results are expressed as means and vertical and horizontal bars show s.d. means.



be significantly greater than that of the R enantiomer in the present study. Consequently the greater potency of S(-) warfarin does not result from the plasma concentrations of this enantiomer. It has been proposed that the greater anticoagulant effect is due to a difference in permeability or affinity for the receptor site (O'Reilly, 1974a). The S enantiomer of warfarin exhibits a higher degree of plasma protein binding in man, and therefore may reflect a higher affinity for the receptor (Banfield et al., 1983).

This receptor site is thought to be the enzyme vitamin K epoxide reductase (Bell and Matschiner, 1972) and the detection of vitamin K_1 2,3-epoxide in the plasma of the volunteers given warfarin enantiomers (1 mg daily) followed by a pharmacological date of vitamin K_1 is consistent with this theory. If inhibition of vitamin K epoxide reductase is related to the anticoagulant effect of 4-hydroxycoumarin anticoagulants, then a greater accumulation of vitamin K_1 2,3-epoxide would be expected with S(-) warfarin than with R(+) warfarin. Although this has been demonstrated in rats (Schmidt et al., 1979; Bell and Ren, 1981), previous studies in man have failed to show any difference in epoxide formation between the two enantiomers (Shearer et al., 1977; Choonara et al., 1986). However, the doses of warfarin used (50 and 15 mg respectively) may have exceeded that required for maximum accumulation of epoxide.

In the present study the enantiomers of warfarin were administered at a dose of 1 mg daily until steady state plasma concentrations were achieved. At this dose a stereoselective effect on the inhibition of vitamin K epoxide reductase was observed. S(-) warfarin produced significantly higher plasma concentrations of vitamin K_1 2,3-epoxide than did R(+) warfarin. This indicates that S(-) warfarin inhibits

the recycling of vitamin K_1 2,3-epoxide back to vitamin K_1 to a greater extent than R(+) warfarin at this dose (1 mg).

As previously mentioned, the results from the present study illustrate significantly higher plasma clearance values for S(-) warfarin than for R(+) warfarin. To compare potency it would have been ideal to have had similar plasma concentrations of the enantiomers. This however is very difficult to achieve because of the inter-individual variation in the plasma clearance of the enantiomers. Nevertheless, the greater potency of S(-) warfarin was demonstrated in the present study despite lower plasma concentrations of this enantiomer by the greater increase in prothrombin time after S(-) warfarin than after R(+) warfarin. From the concentration-response curves obtained for the enantiomers it can be seen that this curve for S(-) warfarin is to the left of that for R(+) warfarin thus illustrating the greater potency of the S enantiomer.

In addition, the results confirm that R(+) warfarin does have a significant effect on clotting factor synthesis as suggested by previous studies (Breckenridge et al., 1974; O'Reilly, 1974a; Wingard et al., 1978).

7.5 Conclusion

The detection of vitamin K_1 2,3-epoxide in the plasma of the volunteers involved in both of the studies described in this chapter is consistent with the hypothesis that the warfarin receptor is associated with the enzyme vitamin K epoxide reductase.

A differential effect between the accumulation of plasma vitamin K_1 2,3-epoxide and change in prothrombin time was observed following low daily doses of warfarin. However, the production of concentration-response curves to investigate the pharmacological effect of warfarin lead to the conclusion that the differential effect was associated with a lack of sensitivity of the prothrombin time assay when compared to the detection of plasma vitamin K_1 2,3-epoxide.

CHAPTER 8

THE DISPOSITION OF A PHARMACOLOGICAL DOSE OF VITAMIN K_1 IN RELATION TO NORMAL PHYSIOLOGICAL CONCENTRATIONS IN MAN AND THE RABBIT.

8.1 Introduction

Vitamin K_1 is an essential co-factor for the post-ribosomal γ-carboxylation of glutamyl residues in clotting factors II, VII, IX and X, during which it is converted to the biologically inactive metabolite vitamin K_1 2,3-epoxide (Jackson and Suttie, 1977). Under normal circumstances the epoxide is recycled back to vitamin K_1 by the enzyme vitamin K epoxide reductase (Willingham and Matschiner, 1974; Matschiner et al., 1974; Zimmerman and Matschiner, 1974). The enzyme is thought to be inhibited in the presence of 4-hydroxy-coumarin anticoagulants and thus clotting factor synthesis is blocked (Bell, 1978).

Until recently, the majority of studies on the disposition of vitamin K_1 and its antagonism by coumarin anticoagulants have utilized either radiolabelled vitamin K_1 (Shearer et al., 1976; Bjornsson et al., 1979; Haustein, 1984) or a pharmacological dose of the vitamin (Hart et al., 1984; Park et al., 1984) which can be quantified using HPLC with ultraviolet detection. However, recent developments in analytical techniques have resulted in the use of HPLC with electrochemical detection (Section 2.3.5). Using this methodology it is possible to determine physiological concentrations of vitamin K_1 in biological material.

The ability of brodifacoum to produce sustained anticoagulation is well documented (Barlow et al., 1982; Park and Leck, 1982), and as a consequence, this novel 4-hydroxycoumarin anticoagulant is a useful experimental tool for the maintenance of vitamin K_1 antagonism in experimental animals.

Using HPLC with electrochemical detection, the aim of the studies described in this chapter was to obtain fundamental informa-

tion regarding the metabolic handling of vitamin K_1 in man and the rabbit, in both the presence and the absence of the 4-hydroxycoumarin anticoagulants, warfarin and brodifacoum.

The rabbit was chosen for these studies because a considerable amount of work on the mechanism of action of coumarin anticoagulants has been carried out in this species (Park et al., 1979; Hart et al., 1984), and also that a large number of blood samples could be collected over a period of up to several weeks. Furthermore liver samples could be obtained, and therefore it was possible to investigate plasma and hepatic concentrations of vitamin K_1, following a pharmacological dose, in relation to normal physiological concentrations of the vitamin.

8.2 Methods

The Disposition of a Pharmacological Dose of Vitamin K_1 in the Rabbit

The physiological plasma concentrations of vitamin K_1 were determined in one group of rabbits (n = 4) at the same time for 5 successive days and in another group of rabbits (n = 4) at the same time for 7 successive days. Blood samples (5 ml) were collected from the right marginal ear vein into heparinised blood tubes. Plasma was obtained by centrifugation (2000 x g; 20 min) and stored frozen (-20°C) until required for analysis as described in section 2.3.5.

At t = -24 h groups of rabbits (n = 6) received single doses of either brodifacoum dissolved in polyethylene glycol 200 (10 mg kg^{-1}; 0.5 ml kg^{-1}) or polyethylene glycol 200 alone (0.5 ml kg^{-1}) via the left marginal ear vein. Vitamin K_1 (Konakion$^{(R)}$; 10 mg kg^{-1}; 1 ml kg^{-1}) was administered via the left marginal ear vein to all rabbits at t = 0 h.

Blood samples (5 ml) were collected into heparinised blood tubes from the right marginal ear vein at t = 0.5, 1, 2, 4, 6, 10, 16, 24, 34, 48, 58, 72, 82, 96, 144, 168, 216 and 264 h after vitamin K_1 administration in control animals and at the same time points up to 77 h in brodifacoum pretreated animals. Plasma was obtained by centrifugation (2000 x g; 20 min) and stored frozen (-20°C) until required for analysis. Samples 0.5 to 16 h were analysed by HPLC with UV detection as described in section 2.3.3 and samples 16 to 77h or 264 h were analysed by HPLC with electrochemical detection as described in section 2.3.5.

Further blood samples (0.9 ml) were collected at t = 0, 2, 6, 10, 24, 48, 72 and 77 h from the anticoagulant pretreated group of rabbits for the immediate determination of prothrombin complex activity (P.C.A.) as described in section 2.3.7.

At t = 77 h, livers (86 to 120g) were removed from the rabbits pretreated with brodifacoum. A portion of the liver (18.2 to 22.6g) was finely chopped and added to phosphate buffer (1 vol). Homogenisation was carried out in a pyrex pestle using a mechanical homogeniser. The homogenate was then stored frozen (-20^{o}C) until required for analysis (section 2.3.6). Livers (76 to 141g) were also removed from an additional group of rabbits (n = 6) which had received no anticoagulant or vitamin K_1. Portions of these livers were taken (17.7 to 24.8g) and prepared for analysis as described above.

The plasma half-lives were calculated from the plasma concentration-time data as described in section 2.4. Results are expressed as mean ± standard deviation of the mean (mean ± s.d.). Levels of significance were determined using the non-paired Student's t test.

The Disposition of a Pharmacological Dose of Vitamin K_1 in Man

Venous blood samples (10 ml) were collected into heparinised blood tubes from six healthy male volunteers and from six of the patients on steady state warfarin therapy involved in the M R C study described in Chapter 6. Plasma was obtained by centrifugation (2000 x *g*; 20 min) and stored frozen (-20^{o}C) until required for the determination of physiological plasma concentrations of vitamin K_1 as described in section 2.3.5.

Using venous blood samples collected from volunteers involved in the studies described in Chapter 7, the plasma disposition of an intravenous dose of vitamin K_1 (10 mg) in the presence of either R(+), S(-) or racemic warfarin at steady state was determined. The samples collected between 0.5 and 12 h following vitamin K_1 administration, were analysed by HPLC with UV detection as described in section 2.3.3, and those collected between 12 and 72 h were analysed by HPLC with electrochemical detection as described in section 2.3.5.

Plasma half-lives were calculated from the plasma concentration-time data as described in section 2.4. Results are expressed as mean $\pm$ standard deviation of the mean (mean $\pm$ s.d.).

8.3 Results

The Disposition of a Pharmacological Dose of Vitamin K_1 in Rabbit

In order to determine the normal range of physiological plasma concentrations of vitamin K_1 in the rabbit, four animals were studied over a period of 7 days and another four animals over a period of 5 days. The results obtained are illustrated in Figure 8.1 and it can be seen that in both groups of rabbits the plasma concentrations of vitamin K_1 remained relatively stable throughout the time studied. The mean plasma concentrations obtained for each animal ranged from 9.8 to 26.5ng ml^{-1}. However, 24 h after the administration of brodifacoum (10 mg kg^{-1}), physiological plasma vitamin K_1 concentrations (8.2 ± 2.3 ng ml^{-1}) were significantly ($P < 0.005$) lower than those in control animals (15.7 ± 3.9 ng ml^{-1}) (Figure 8.3). At this time, P.C.A. had been markedly reduced to $24.9 \pm 4.4\%$ in the brodifacoum pretreated animals (Figure 8.2). However, following administration of a pharmacological dose of vitamin K_1 (10 mg kg^{-1}), P.C.A. increased sharply, reaching a maximum ($129 \pm 8.7\%$) between 2 and 6 h. P.C.A. then declined with a half-life of degradation of 10.7 ± 3.7 h.

Figure 8.3 illustrates the plasma concentration data for vitamin K_1 obtained for control and brodifacoum pretreated animals. The results obtained for the samples collected at t = 16 h were very similar by both detection methods employed. For example, at this time the plasma concentration of vitamin K_1 in rabbit 7 was found to be 151 ng ml^{-1} by UV detection and 155 ng ml^{-1} by electrochemical detection. It can be seen that in both groups of rabbits, there is a very rapid decline in vitamin K_1 plasma concentrations between 0.5 and 2 h. Previous work has shown this to be the α-phase

FIGURE 8.1 Endogenous (physiological) plasma concentrations of vitamin K_1 in rabbits over a period of 7 or 5 successive days.

Results are expressed as means (n = 4) and vertical bars show s.e. mean.

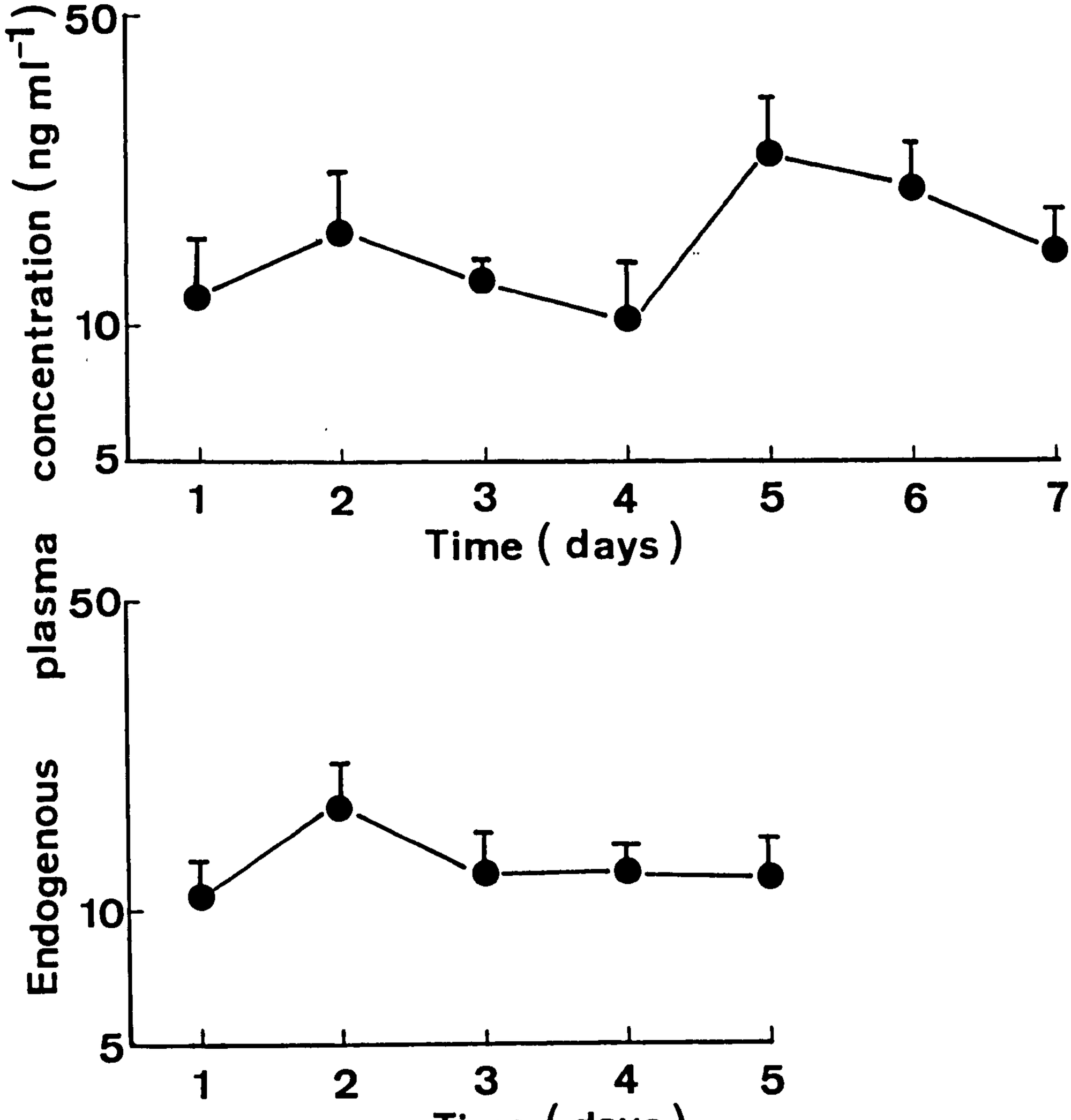


FIGURE 8.2 Prothrombin complex activity (P.C.A.) vs. time in rabbits 24 h after intravenous administration of brodifacoum (10 mg kg^{-1}) and following intravenous administration of vitamin K_1 (10 mg kg^{-1}) at t = 0 h.

Results are expressed as means (n = 6) and vertical bars show s.e. mean.

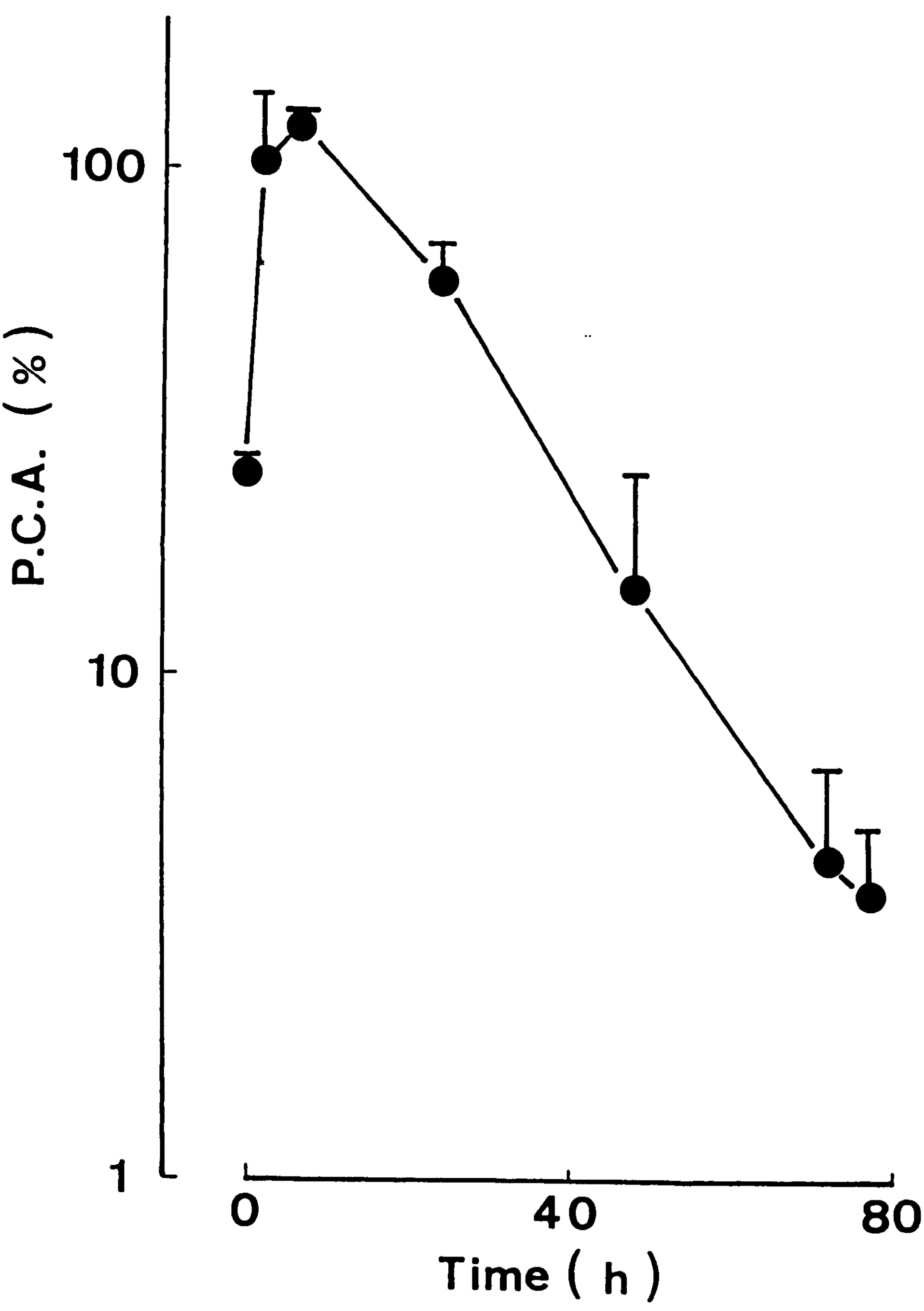
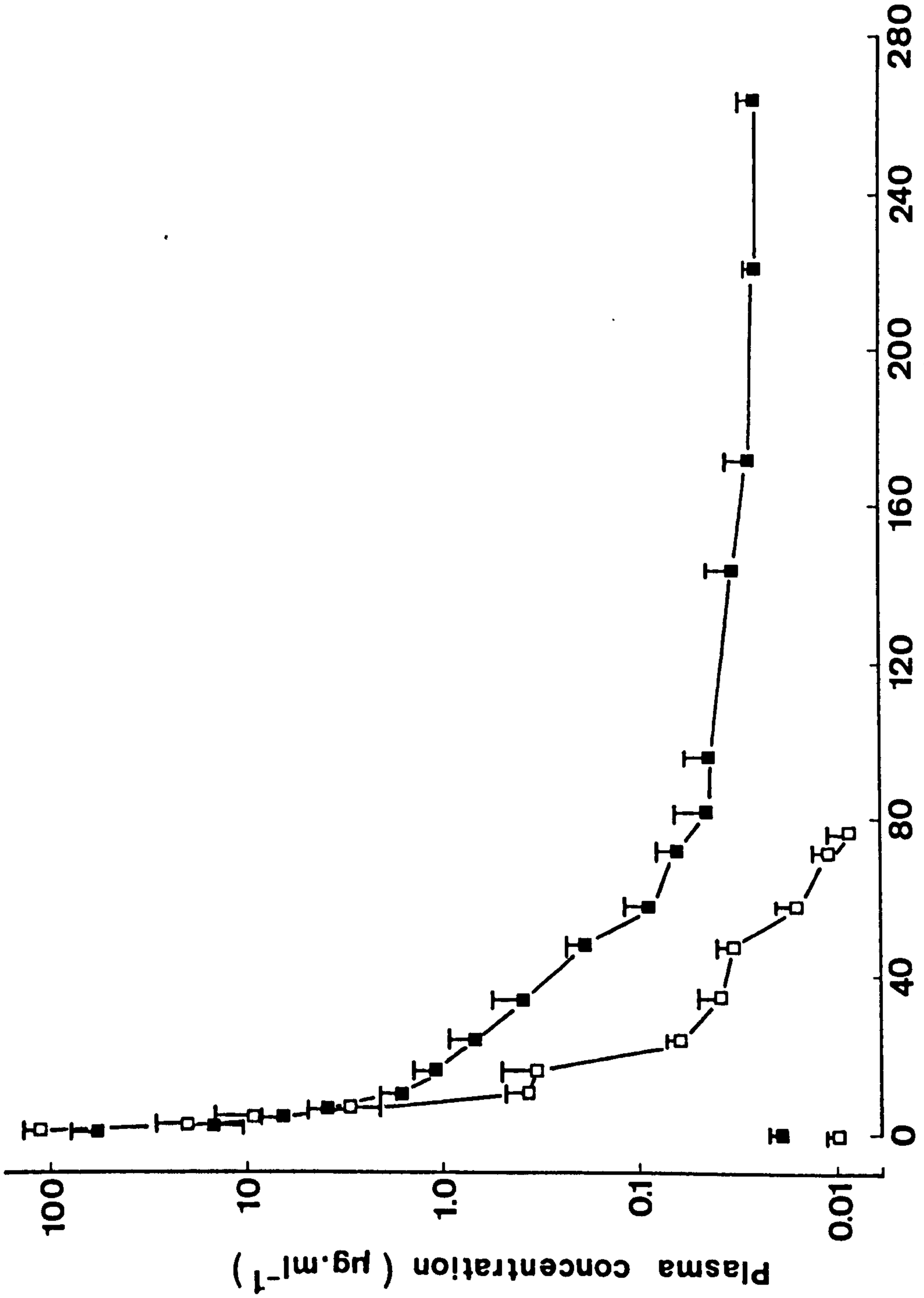


FIGURE 8.3 Plasma concentrations of vitamin K_1 vs. time in control rabbits (■) before and after intravenous administration of vitamin K_1 (10 mg kg^{-1}) at t = 0 h and in brodifacoum pretreated rabbits (□) 24 h after intravenous administration of brodifacoum (10 mg kg^{-1}) and after intravenous administration of vitamin K_1 (10 mg kg^{-1}) at t = 0 h. Results are expressed as means (n = 6) and vertical bars show s.e. mean.


Plasma concentration (μg.ml⁻¹)
100
10
1.0
0.1
0.01
0
40
80
120
160
200
240
280
Time (h)

of vitamin K_1 elimination and the half-life has been calculated to be less than 1 h (Park et al., 1980; Hart et al., 1984). Although it has been demonstrated that the rabbit is a useful animal for use in studies which require the collection of regular blood samples over a prolonged period of time, there is a limitation on the amount of blood that can actually be taken. As the present study was concerned with the subsequent phases of vitamin K_1 elimination, insufficient blood samples were collected during the two hours following vitamin K_1 administration to determine an α-half life. The α-phase of vitamin K_1 - elimination is followed by a rapid β-phase in both groups of animals. Figure 8.3 illustrates the occurrence of the β-phase between 2 and 10 h in both groups of animals. Statistical analysis revealed no significant difference in β-half-life between the two groups (Table 8.1).

Following the β-phase, both groups of animals displayed a much slower phase of elimination of the vitamin, which will be referred to as the γ-phase. There was no significant difference in the γ-half-life between the two groups of animals (Table 8.1). Blood samples were only collected until t = 77 h from the brodifacoum pretreated animals because at this time P.C.A. was $\leqslant$ 5% and as a consequence the animals were sacrificed.

The vitamin K_1 content of whole liver homogenate was significantly ($P < 0.01$) lower in the brodifacoum pretreated animals (44.1 ± 18.4 ng g^{-1}) than in a group of animals which had received neither vitamin K_1 nor anticoagulant, i.e. normal physiological concentrations (127.7 ± 44.3 ng g^{-1}).

TABLE 8.1 Plasma half-lives of vitamin K_1 in control and brodifacoum pretreated rabbits following a pharmacological dose of the vitamin (10 mg kg^{-1}).

Control

Rabbit	$\beta - t_{\frac{1}{2}}$ (h)	$\gamma - t_{\frac{1}{2}}$ (h)
1	1.10	14.55
2	1.29	17.34
3	1.09	12.99
4	3.11	13.67
5	1.33	12.19
6	1.34	20.02
mean	1.54	15.13
$\pm$ s.d.	0.78	2.98

Brodifacoum

Rabbit	$\beta - t_{\frac{1}{2}}$ (h)	$\gamma - t_{\frac{1}{2}}$ (h)
7	1.06	9.87
8	1.20	19.80
9	1.32	8.46
10	0.67	9.62
11	0.74	19.37
12	0.72	13.63
mean	0.95	13.46
$\pm$ s.d.	0.28	5.06

The Disposition of a Pharmacological Dose of Vitamin K_1 in Man

Table 8.2 illustrates the individual and the mean physiological plasma vitamin K_1 concentration data obtained for healthy volunteers and for patients on steady state warfarin therapy. Statistical analysis revealed no significant difference between the values obtained for the two groups. Figure 8.4 illustrates the bi-exponential nature of the elimination of a pharmacological dose of vitamin K_1 (10 mg) in one of the healthy male volunteers (MT) involved in this study. This was typical for all volunteers receiving daily anticoagulant therapy (1 mg) of one of the enantiomers of warfarin or racemic warfarin.

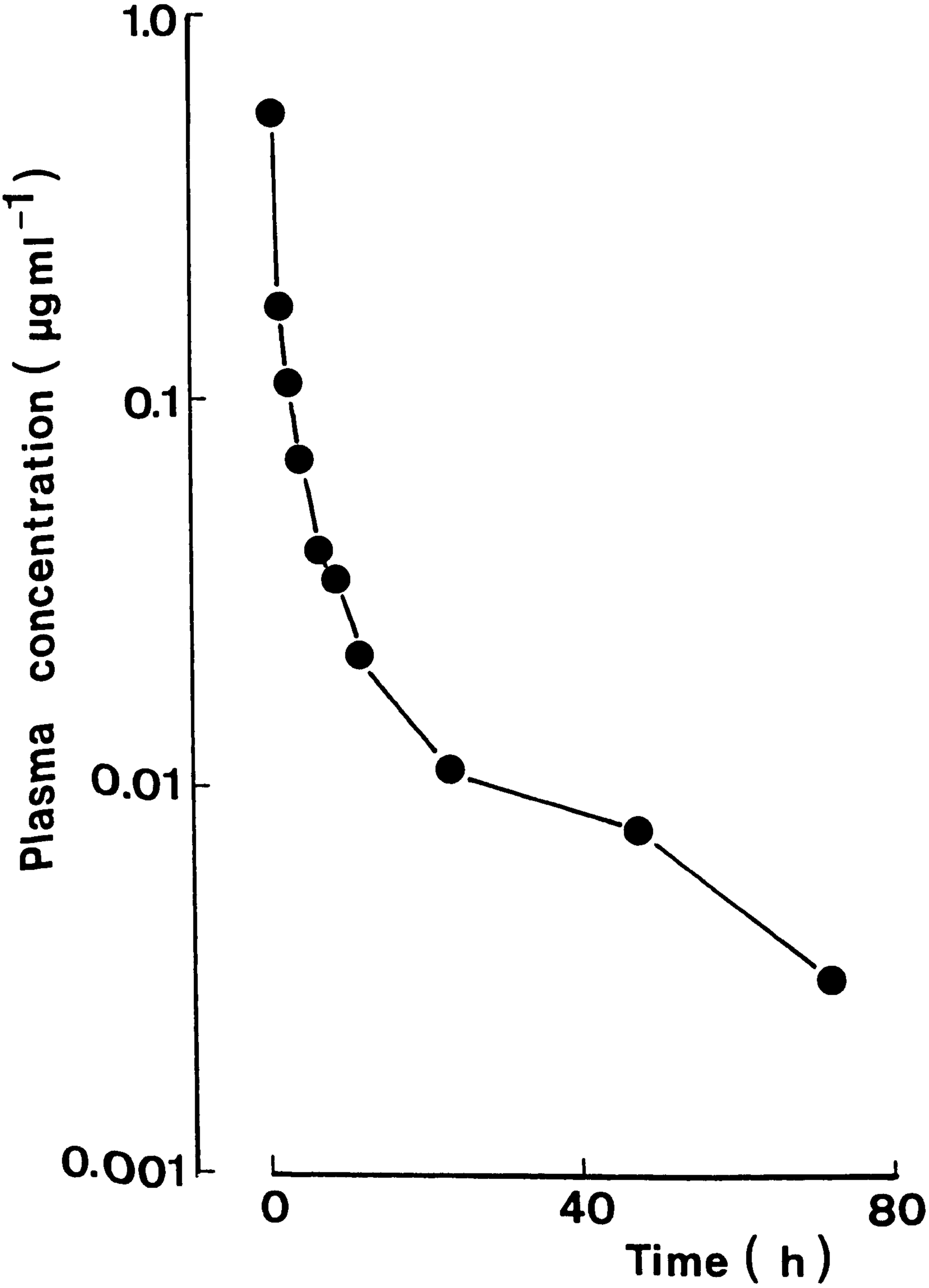
Insufficient blood samples were collected from the volunteers during the 0 to 2 h period following vitamin K_1 administration to allow analysis of the α-phase of elimination. This phase has previously been documented by other workers (Shearer et al., 1977; Bjornsson et al., 1979). The initial phase of elimination determined in this study is referred to as the β-phase and this is followed by the γ-phase. The β-half-life was found to range from 1.4 to 5.9 h and the γ-half-life from 16.3 to 37.1 h.

TABLE 8.2 Endogenous vitamin K_1 concentrations in the plasma of volunteers and patients on steady state warfarin

Volunteer	Vitamin K_1 ($ng\ ml^{-1}$)
SB	1.70
IB	1.59
RC	1.53
AD	1.25
PR	2.04
MT	1.08
mean $\pm$ s.d.	1.53 ± 0.34

Patient	Vitamin K_1 ($ng\ ml^{-1}$)
BC	3.78
RD	2.07
DE	1.87
IM	1.92
WS	1.32
CW	2.45
mean $\pm$ s.d.	2.24 ± 0.84

FIGURE 8.4 Plasma concentrations of vitamin K_1 vs. time in a single individual (MT) on steady state racemic warfarin therapy (1 mg daily) following intravenous administration of vitamin K_1 (10 mg) at t = 0h.



8.4 Discussion

During the production of active clotting factors II, VII, IX and X, vitamin K_1 is converted to vitamin K_1 2,3-epoxide which under normal circumstances is converted back to the vitamin by the enzyme vitamin K epoxide reductase. This continual recycling of vitamin K_1 is known as the vitamin K epoxide cycle. The vitamin K_1 utilized in this biochemical system is thought to be contained in what is known as the body pool of vitamin K. The size of the pool has been estimated to be about 100 μg kg^{-1} of liver (Duello and Matschiner, 1972; Bjornsson et al., 1979) and is thought to be associated with the rough endoplasmic reticulum of hepatocytes, as this is where the enzyme activities associated with the vitamin K epoxide cycle are located (Carlisle and Suttie, 1980).

In the present study physiological plasma concentrations of vitamin K_1 have been determined in both the rabbit and in man. Each rabbit involved in the study was monitored for several successive days to determine any fluctuations in physiological plasma vitamin K_1 content. The samples were taken at the same time each day so as to avoid any possible diurnal variation in vitamin K_1 concentration, and were found to be relatively stable throughout the time studied. The inter-animal variation (9.8 ± 3.8 to 26.5 ± 10.9 ng ml^{-1}) may reflect differences in food intake and coprophagy. These values can be seen to be approximately ten-fold greater than those determined for a group of healthy male volunteers (1.53 ± 0.34 ng ml^{-1}), illustrating a species difference in physiological plasma vitamin K_1 concentrations.

4-hydroxycoumarin anticoagulants such as brodifacoum and warfarin are thought to produce their pharmacological effect by inter-

ruption of the vitamin K epoxide cycle at the enzyme vitamin K epoxide reductase (Bell and Matschiner, 1972; Whitlon et al., 1978; Park et al., 1979). This causes a decrease in clotting factor synthesis and a subsequent anticoagulant effect.

Initially the effect of brodifacoum on plasma concentrations of vitamin K_1 was investigated. 24 h following the administration of brodifacoum to rabbits at a dose 10 times greater than that which had previously been shown to produce maximum inhibition of vitamin K_1 for at least 7 days (Park and Leck, 1982), physiological plasma concentrations of vitamin K_1 were significantly lower ($P < 0.001$) than those in the control group of animals. This indicates that brodifacoum has the ability to reduce plasma vitamin K_1 concentrations to below normal physiological concentrations. In contrast, no significant difference was found between the physiological plasma concentrations of vitamin K_1 in volunteers on steady state warfarin and those individuals in whom coumarin anticoagulants were absent. This may be due to the fact that the dose of warfarin administered was not large enough to produce sufficient inhibition of the enzyme vitamin K epoxide reductase to exhaust the body pool of vitamin K_1 or that plasma concentrations of vitamin K_1 do not reflect concentrations of vitamin at its site of action. 0.5 h following the administration of a pharmacological dose of vitamin K_1 to both control and brodifacoum pretreated rabbits, the plasma concentrations of the vitamin were 10,000 times greater than those determined as normal physiological concentrations. At the same time as these huge plasma concentrations of vitamin K_1, there was an immediate sharp rise in P.C.A. in the brodifacoum pretreated animals. This phenomenon has previously been attributed to the rapid γ-carboxyla-

tion of accumulated clotting factors (Suttie, 1970), and indicates that the vitamin K_1 from the pharmacological dose is available for clotting factor synthesis. However, 2 to 6h following the immediate rise in P.C.A., this was seen to decline rapidly, even though plasma concentrations of the vitamin were between 1000 and 2500 times greater than those of normal physiological plasma concentrations (Figure 8.3). This indicates that during coumarin anticoagulation with brodifacoum, the high plasma concentrations of vitamin K_1 present 2 to 6h following the administration of a pharmacological dose of the vitamin, are not available to support clotting factor synthesis in the liver. This may be due to the fact that for some reason it is not possible for vitamin K_1 from a pharmacological dose to gain access to the site of clotting factor synthesis. Alternatively, the vitamin K_1 may have access to hepatocytes, however it may not be present in sufficient quantities to drive clotting factor synthesis in the presence of brodifacoum, since once it is converted to vitamin K_1 2,3-epoxide it cannot be recycled to vitamin K_1 due to the coumarin inhibition of vitamin K epoxide reductase.

In most of the previous studies concerned with the pharmacokinetics of vitamin K_1 in man and the rabbit, only the α- and the β-phase of elimination could be determined due to the limitations of the analytical methods employed. The α-phase has been shown to be unaffected by coumarin anticoagulation and to occur with a half-life of approximately 30 min during the first two hours following intravenous vitamin K_1 administration to both man (Shearer et al., 1977; Park et al., 1984) and the rabbit (Hart et al., 1984).

In the present animal study, the β-phase of the elimination of plasma vitamin K_1, was seen to occur between 2 and 10 h in both the

brodifacoum pretreated and the control rabbits. No significant difference in the β-half-lives between the two groups was observed and they were similar to those determined in previous studies (Park et al., 1980; Hart et al., 1984). Likewise, the β-half-lives determined for the individuals involved in the present study, were similar to those determined in previous studies in both the presence (Shearer et al., 1974; 1.9 to 4.3 h) and the absence of 4-hydroxycoumarin anticoagulants (Park et al., 1984; 1.4 to 2.2 h). Thus the data is in agreement with the findings of previous studies which suggest that coumarin anticoagulants have no effect on the β-phase of vitamin K_1 elimination (Shearer et al., 1977; Park et al., 1980).

Following the β-elimination phase of vitamin K_1, a γ-phase was observed in both groups of rabbits. In the control group of animals, the γ-phase of elimination continued until 82 h following vitamin K_1 administration at which point plasma concentrations of the vitamin were not significantly different to physiological concentrations. The γ-phase of vitamin K_1 elimination continued until the brodifacoum pretreated animals were sacrificed as a result of P.C.A. being $\leqslant$ 5% in these animals. At this time, the plasma concentrations of vitamin K_1 (7.0 ± 4.0 ng ml^{-1}) were very similar to the sub-normal concentrations determined 24 h following brodifacoum administration (8.2 ± 2.0 ng ml^{-1}). There was no significant difference in the γ-half-life between the anticoagulated and the control animals.

Following the β-phase of vitamin K_1 elimination in man, there was a considerably longer γ-phase with a plasma half-life of 16.3 to 37.1 h. This phase of vitamin K_1 elimination has not previously been reported in man. The final sample taken from the individuals on steady state

warfarin contained a plasma concentration of vitamin K_1 (0.76 to 3.08 ng ml^{-1}) very similar to that determined as the normal physiological range in man (1.08 to 2.04 ng ml^{-1}). Thus the γ-phase of vitamin K_1 elimination in control and coumarin anticoagulated rabbits. and in volunteers on steady state warfarin therapy resulted in the return to physiological plasma concentrations of the vitamin.

Brodifacoum has been shown to significantly reduce physiological plasma concentrations of vitamin K_1 in the rabbit over a period of 24 h. However, following a pharmacological dose of vitamin K_1, no significant difference in the pharmacokinetics of the vitamin was determined between those animals pretreated with brodifacoum and those which had received vitamin K_1 alone. Furthermore, 77 h following the administration of the pharmacological dose of vitamin K_1, plasma concentrations of the vitamin in the brodifacoum pretreated animals were very similar to the sub-normal concentrations determined prior to vitamin K_1 administration.

To gain more direct information concerning vitamin K_1 at its site of action in the liver, the vitamin K_1 content of whole liver homogenate was determined in the group of animals pretreated with brodifacoum and then given a pharmacological dose of vitamin K_1, and in an additional group of rabbits which received neither vitamin K_1 nor anticoagulant. Thus the vitamin K_1 content of liver taken from this latter group of animals was solely physiological. The results demonstrated a four-fold greater hepatic concentration of vitamin K_1 in this additional control group of rabbits than in brodifacoum pretreated animals which had received a pharmacological dose of the vitamin. Results obtained in the rat (Chapter 9) indicate that hepatic concentrations of vitamin K_1 after a pharmacological dose of

the vitamin to animals which had received no anticoagulant pretreatment, would be approximately six times greater than those which had received brodifacoum pretreatment.

Brodifacoum has been shown to have the ability to deplete the body pool of vitamin K_1 to below normal physiological concentrations, as reflected by brodifacoum pretreatment to rabbits causing decreased physiological plasma and hepatic concentrations of the vitamin. However, despite the discovery of a third phase (γ-phase) of vitamin K_1 elimination following the administration of a pharmacological dose of the vitamin, brodifacoum pretreatment had no significant effect on the pharmacokinetics of vitamin K_1. Thus the pharmacokinetics of a pharmacological dose of vitamin K_1 do not reflect concentrations of vitamin K_1 at its site of action in the liver, whereas physiological plasma concentrations do.

8.5 Conclusions

The rapid decline in P.C.A. at a time when plasma concentrations of vitamin K_1 were 1000 to 2500 times greater than those of normal physiological plasma concentrations, illustrates the lack of simple equilibrium between vitamin K_1 in plasma and vitamin K_1 in the liver of brodifacoum anticoagulated rabbits.

The ability of brodifacoum to produce a depletion of both physiological plasma and hepatic concentrations of vitamin K_1, and the lack of significant difference in the pharmacokinetics of a pharmacological dose of vitamin K_1 between control and brodifacoum pretreated animals, indicates that despite numerous attempts in the literature to use plasma pharmacokinetics of vitamin K_1 to monitor the vitamin at its site of action in the liver, these results, complete with the investigation of the previously unknown γ-phase of elimination, illustrate that this approach cannot be used.

CHAPTER 9

A STUDY OF THE DISPOSITION OF A PHARMACOLOGICAL DOSE OF VITAMIN K_1 IN RELATION TO PHARMACOLOGICAL RESPONSE IN THE RAT

9.1 Introduction

Patients poisoned with coumarin anticoagulants have been shown to require frequent and repeated pharmacological doses of vitamin K_1 over a long period of time to restore clotting factor synthesis (Barlow et al., 1982; Jones et al., 1984a; Lipton and Klass, 1984). This is supported by the finding that in brodifacoum anticoagulated rabbits, a pharmacological dose of vitamin K_1 (10 mg kg^{-1}) has a short duration of action (2 to 6 h) and that high plasma concentrations of vitamin K_1 (ca 10 µg ml^{-1}) are required to drive clotting factor synthesis (Chapter 8). In contrast, it has been shown that the daily requirement of vitamin K_1 in man is low (ca 1 µg kg^{-1}; Frick et al., 1967; Barkhan and Shearer, 1977) and that circulating concentrations of vitamin K_1 are low (1.08 to 2.04 ng ml^{-1}; Chapter 8: 0.8 to 1.8 ng ml^{-1}; Haroon et al., 1984). Thus there appears to be a huge difference between physiological plasma concentrations of vitamin K_1 and plasma concentrations required in coumarin overdose.

At present there is no standard regime for the administration of vitamin K_1 as an antidote to coumarin poisoning, and as a consequence several studies, including that described in Chapter 8, have been carried out to explain the short duration of action of vitamin K_1 in coumarin poisoning by monitoring plasma concentrations of the vitamin (Hart et al., 1984; Park et al., 1984). This work indicated that vitamin K_1 plasma concentration data has limited value in furthering our knowledge of vitamin K_1 and its antagonism by coumarin anticoagulants. This prompted an investigation of the effect of chronic anticoagulation on hepatic concentrations of vitamin K_1 and its metabolite vitamin K_1 2,3-epoxide.

The aim of this study was two-fold. Firstly to develop the rat as a viable animal model in which the pharmacology of vitamin K_1 and its antagonism by 4-hydroxycoumarin anticoagulants could be investigated. The second aim was to use the animal model to determine the relationship between hepatic and plasma concentrations of vitamin K_1 and vitamin K_1 2,3-epoxide, and the pharmacological response to the vitamin in the anticoagulated rat.

9.2 Development of the Animal Model

In Chapters 3 and 8, the development of the rabbit as an animal model in which both the pharmacokinetics and the pharmacodynamics of racemic warfarin and its enantiomers could be determined simultaneously in the same animal, and in which the pharmacokinetics of a pharmacological dose of vitamin K_1 could be determined over a period of several weeks, was described. The rabbit proved useful for these purposes because it is possible to take sequential blood samples from a single conscious animal over a long period of time. However, the aims of the present study were different, and as a consequence the animal had to fulfil a different set of criteria.

A considerable amount of work concerned with the metabolism of vitamin K_1 and its antagonism by coumarin anticoagulants has been carried out in the rat (Willingham and Matschiner, 1974; Ren *et al.*, 1977; Leck and Park, 1981). In addition, many *in vitro* studies concerned with the characterisation and purification of the enzymes involved in the vitamin K epoxide cycle have employed rat hepatic microsomal preparations (Hildebrandt *et al.*, 1984; Kappel and Olsen, 1984; Lee and Fasco, 1984), thus providing a great deal of information on the rat with respect to vitamin K_1 and coumarin anticoagulants.

The studies described in this chapter were partly concerned with the determination of the pharmacological response to vitamin K_1 in the anticoagulated rat, and therefore blood samples had to be taken over a 7 h period to investigate the nature of the response. The method by which the pharmacological response was determined (prothrombin complex activity, P.C.A.; section 2.3.7) requires the rapid collection of a blood sample with as little contact as possible with external surfaces. For this reason blood was collected from the anaesthetised

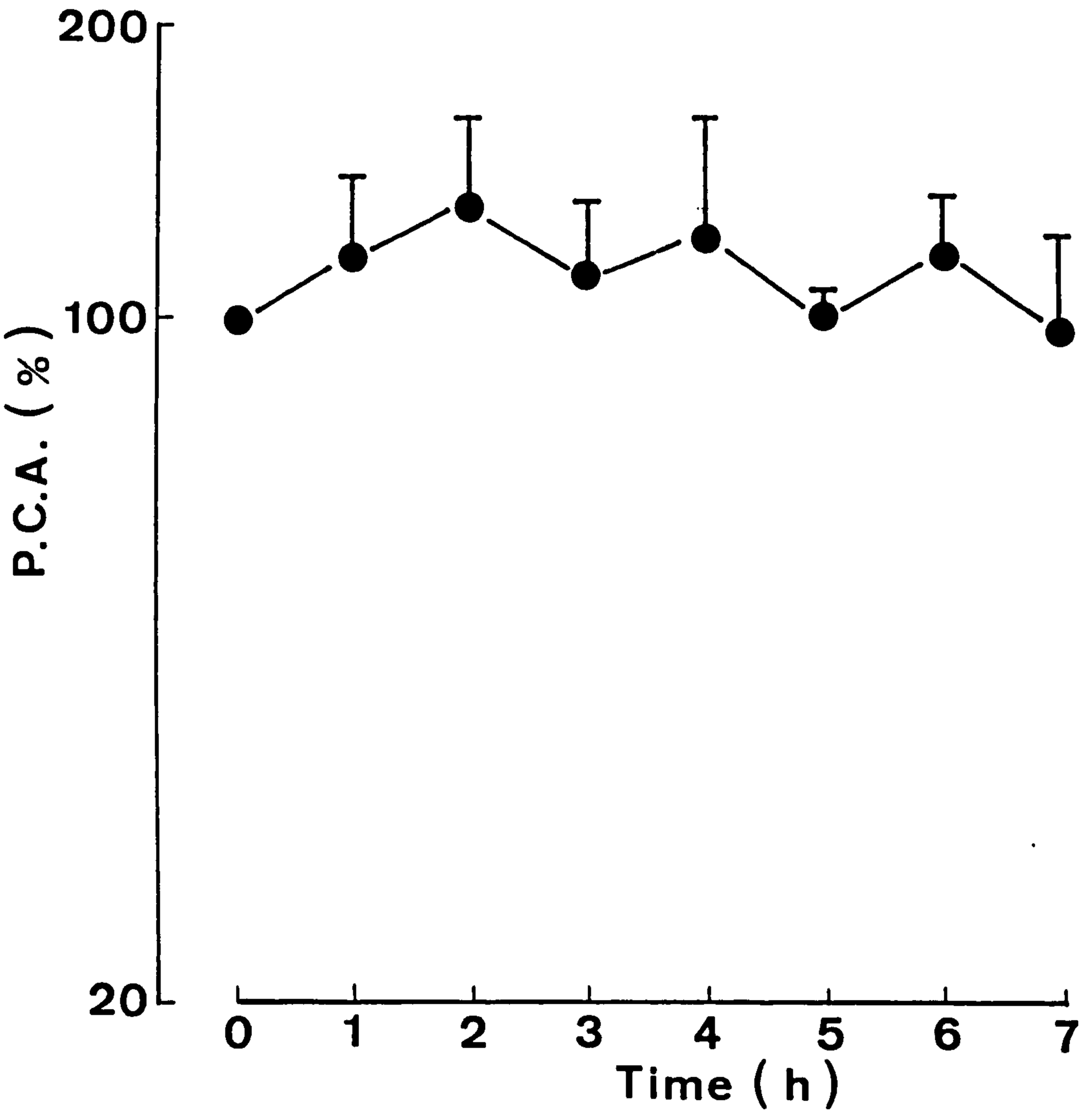
rat via an indwelling cannula in the carotid artery. To maintain cannula patency, immediately after obtaining a blood sample, the cannula was filled with heparinised saline solution (100 iu ml^{-1}) up to the point at which the cannula joined the artery. Great care had to be taken to ensure that the heparinised saline did not enter the blood stream of the animal as this would have affected P.C.A. measurements. As a consequence, a series of experiments had to be carried out in which the amount of heparinised saline allowed to enter the cannula without affecting P.C.A., was determined. Control rats were anaesthetised (section 9.3) and blood samples were obtained via an arterial cannula for the determination of P.C.A. over a period of 7 h. From Figure 9.1 it can be seen that the presence of heparinised saline (100 iu ml^{-1}) in the cannula had no effect on P.C.A. during the time studied, however,because of the presence of heparin in the arterial cannula, the first ten drops of blood expelled from the animal were discarded before a sample was collected.

Extreme care had to be taken to prevent excessive blood loss when surgery was being performed, especially from animals pretreated with coumarin anticoagulants. The minimum amount of blood required to determine P.C.A. was collected from each rat at each time point so as not to decrease the blood volume and its constituents to such an extent that the disposition of vitamin K_1 was affected. After collection of a blood sample, an equal volume of saline was administered via an indwelling venous cannula to maintain a constant blood volume. This cannula also served as the route by which vitamin K_1 was administered in these experiments.

Once the viability of the animal model had been determined, the aim of the present study was to determine the hepatic and plasma

FIGURE 9.1 Prothrombin complex activity (P.C.A.) vs. time in rats with an indwelling arterial cannula containing heparinised saline (100 iu ml^{-1})

Results are expressed as means (n = 6) and vertical bars show s.d. mean.



concentrations of vitamin K_1 and vitamin K_1 2,3-epoxide at the time at which the pharmacological response to vitamin K_1 was maximum in anticoagulated rats. The rat is a useful animal for experiments in which hepatic concentration data is to be obtained, since they are relatively inexpensive and in this type of experiment, many animals have to be sacrificed in order to obtain few data points.

9.3 Methods

Initially a control blood sample (0.54 ml) was taken from the tail vein of each rat, while under light ether anaesthesia, using a wide bore needle (19 G) for the determination of P.C.A. as described in section 2.3.7. At t = -6 h, each group (n = 6) of conscious rats received single intraperitoneal injections of either brodifacoum (10 mg kg^{-1}) or racemic warfarin (63 mg kg^{-1}). Racemic warfarin was administered in 0.9% w/v NaCl solution (1 ml kg^{-1}). Brodifacoum was administered dissolved in polyethylene glycol 200 (1 ml kg^{-1}).

Immediately prior to vitamin K_1 administration, the animals were anaesthetised with urethane (14% w/v solution; 1 ml 100 g^{-1} i.p.). The carotid artery and jugular vein of each animal were cannulated using pp50 polypropylene tubing, for the collection of blood samples and the administration of vitamin K_1 respectively.

A blood sample was collected directly before the administration of vitamin K_1 for the immediate determination of P.C.A. as described in section 2.3.7. Vitamin K_1 was administered either as supplied (10 mg ml^{-1}) or was diluted in 0.9% w/v NaCl solution (1 mg ml^{-1}). At t = 0h vitamin K_1 was administered (0.5, 1.0 or 10 mg kg^{-1}; 0.5 - 1.0 ml kg^{-1}). The venous cannula was then flushed through with 0.9% w/v NaCl solution (0.5 ml) to ensure complete administration. Serial blood samples (0.54 ml) were then collected from the arterial cannula for determination of P.C.A. every hour up to 7 h.

This experiment was then repeated in three further groups of rats (n = 6). Each was pretreated with either brodifacoum (10 mg kg^{-1}), racemic warfarin (63 mg kg^{-1}) or 0.9% w/v NaCl solution (1 ml kg^{-1}).

Surgery was performed as described above, and P.C.A. was determined immediately prior to vitamin K_1 administration at t = 0 h and every hour up to t = 3 h. At this time an additional blood sample (3 to 5 ml) was collected via the arterial cannula into a heparinised blood tube. Plasma was obtained by centrifugation (2000 x g; 20 min) and stored frozen (-20°C) until required for analysis. Plasma concentrations of vitamin K_1 and vitamin K_1 2,3-epoxide were determined as described in section 2.3.3.

Following the collection of the final blood sample, a pp50 polypropylene tubing cannula was inserted into the hepatic portal vein and the liver was perfused with phosphate buffer (2 ml). The liver (8.3 to 10.3 g) was subsequently removed, finely chopped and added to phosphate buffer (2 vols). Homogenisation was carried out using a pyrex pestle and a mechanical homogeniser. The homogenate was then stored frozen (-20°C) until required for analysis. Hepatic concentrations of vitamin K_1 and vitamin K_1 2,3-epoxide were determined as described in section 2.3.4.

Results are expressed as mean $\pm$ standard deviation of mean (mean $\pm$ s.d.). Levels of significance were determined using the Student's non-paired t-test.

9.4 Results

In an initial experiment, the dose of brodifacoum required to produce maximum inhibition of clotting factor synthesis in the rat was determined. Previous work (Park and Leck, 1982) has shown that a single intravenous dose of brodifacoum (10 mg kg^{-1}) produces maximum antagonism of vitamin K_1 (1 mg kg^{-1}) in the rabbit for at least one week. The pharmacological response to vitamin K_1 (1 mg kg^{-1}) in rats anticoagulated with brodifacoum (2 mg kg^{-1} and 10 mg kg^{-1}) is shown in Figure 9.2. It can be seen that in both groups of rats, P.C.A. reaches a maximum 3 h after administration of vitamin K_1, and then declines at a rate indicative of maximum inhibition of clotting factor synthesis (Leck and Park, 1981). The half-lives of degradation of P.C.A. in rats anticoagulated with brodifacoum at 2 mg kg^{-1} and 10 mg kg^{-1} were 2.36 $\pm$ 0.34 h and 2.95 $\pm$ 0.80 h respectively. Therefore, brodifacoum at a dose of 10 mg kg^{-1} can be used to achieve maximum antagonism of vitamin K_1. A comparable degree of antagonism of vitamin K_1 by warfarin could only be achieved with ten times the molar equivalent dose of brodifacoum. Figure 9.3 illustrates the pharmacodynamic response to vitamin K_1 (1 mg kg^{-1}) in rats anticoagulated with either brodifacoum (10 mg kg^{-1}) or warfarin (63 mg kg^{-1}). It can be seen that the response as determined by P.C.A. is very similar in both groups of animals at all the time points studied.

Groups of rats anticoagulated with brodifacoum (10 mg kg^{-1}) were used to investigate the duration of action of different intravenous doses of vitamin K_1. Figure 9.4 illustrates the pharmacological response to vitamin K_1 (0.5, 1.0 and 10 mg kg^{-1}). It can be seen that a single 0.5 mg kg^{-1} dose of vitamin K_1 had no observable pharmacodynamic effect in brodifacoum anticoagulated rats, as P.C.A. declined

FIGURE 9.2 Prothrombin complex activity (P.C.A.) vs. time in rats following intravenous administration of vitamin K_1 (1 mg kg^{-1}) 24 h after intraperitoneal administration of 10 mg kg^{-1} brodifacoum (☆) or 2 mg kg^{-1} brodifacoum (○).

Results are expressed as means (n = 6) and vertical bars show s.d. mean.

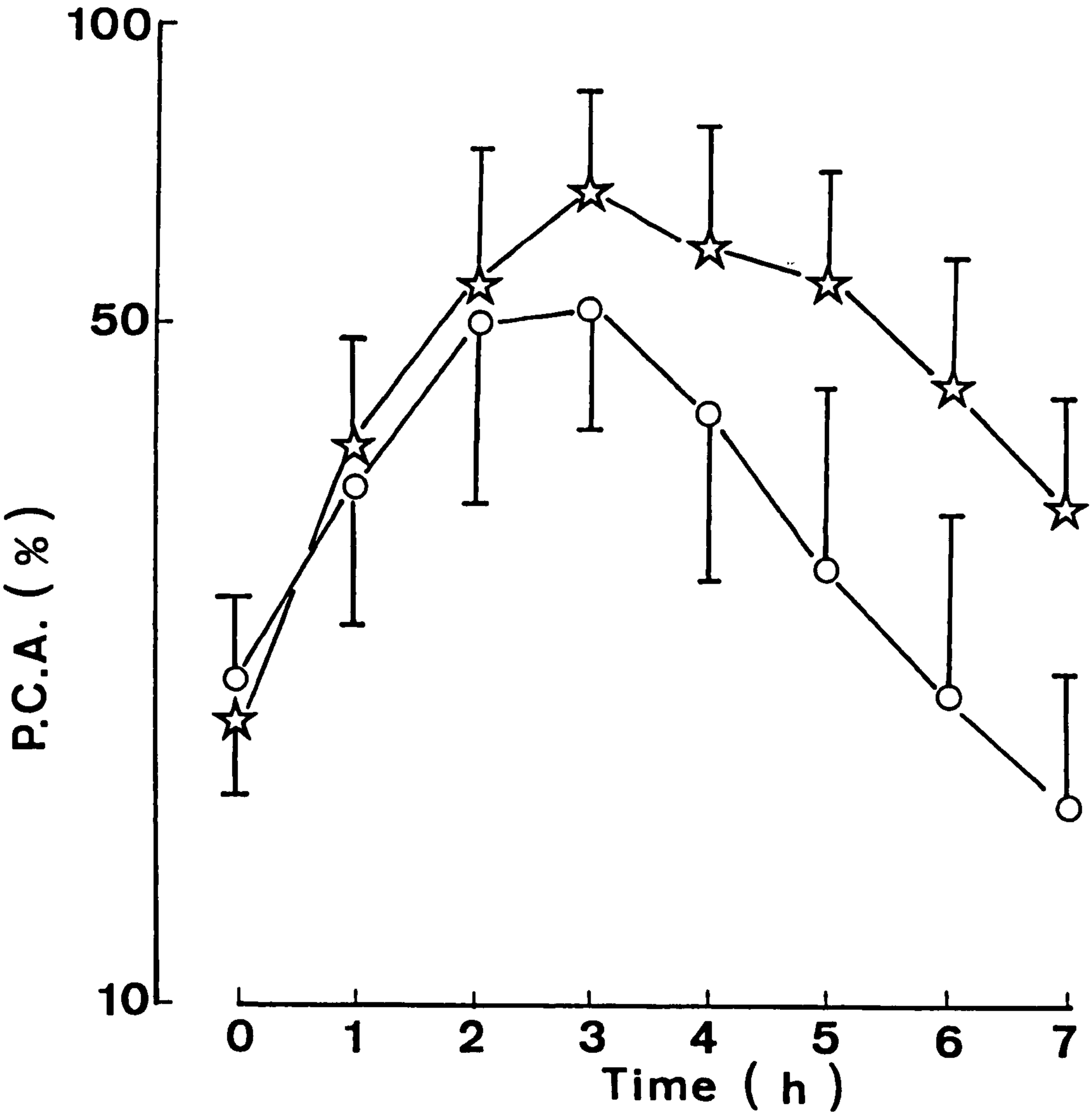


FIGURE 9.3 Prothrombin complex activity (P.C.A.) vs. time in rats following intravenous administration of vitamin K_1 (1 mg kg^{-1}) 24 h after intraperitoneal administration of 10 mg kg^{-1} brodifacoum (☆) or 63 mg kg^{-1} warfarin (★).

Results are expressed as means (n = 6) and vertical bars show s.d. mean.

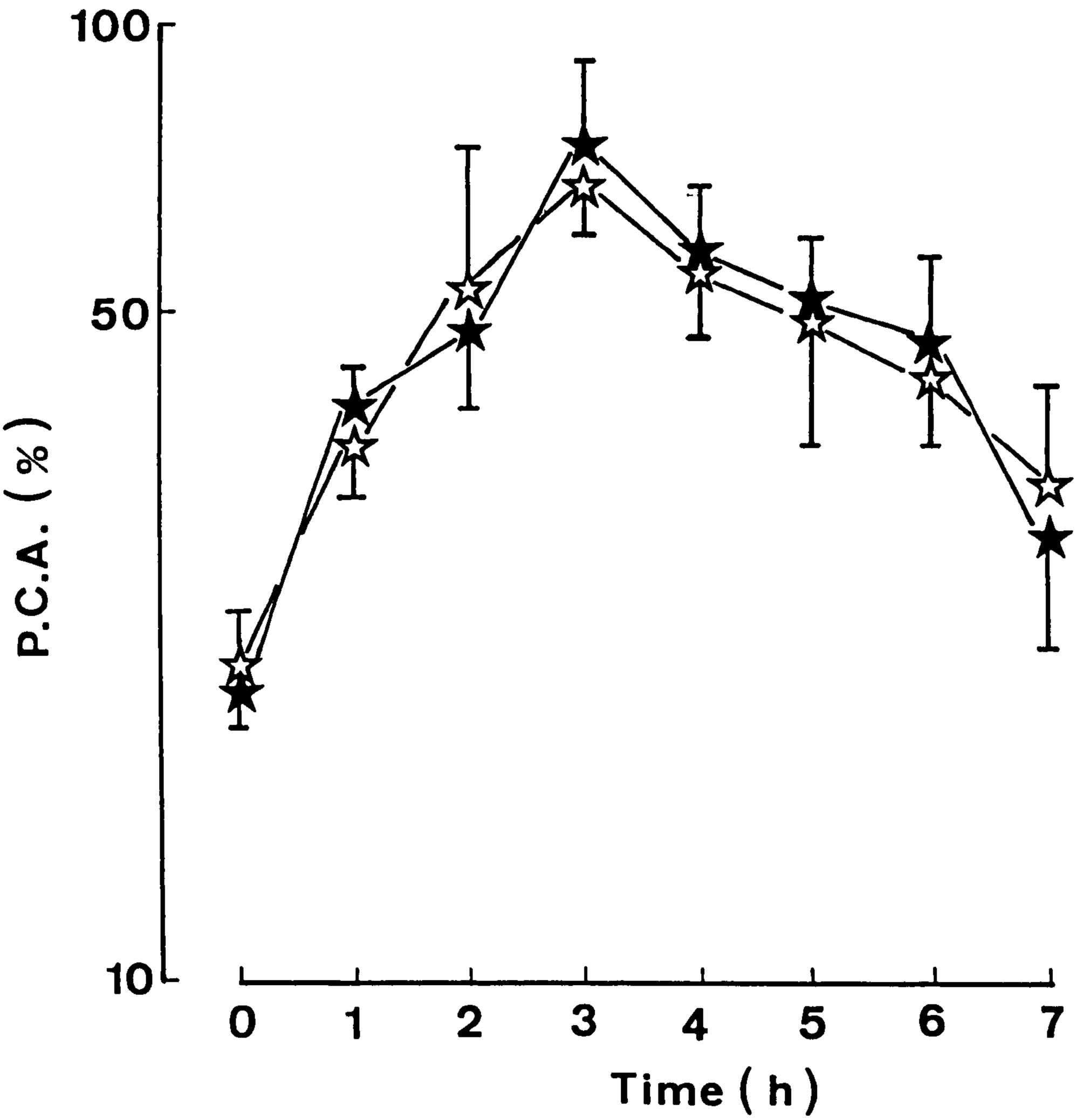
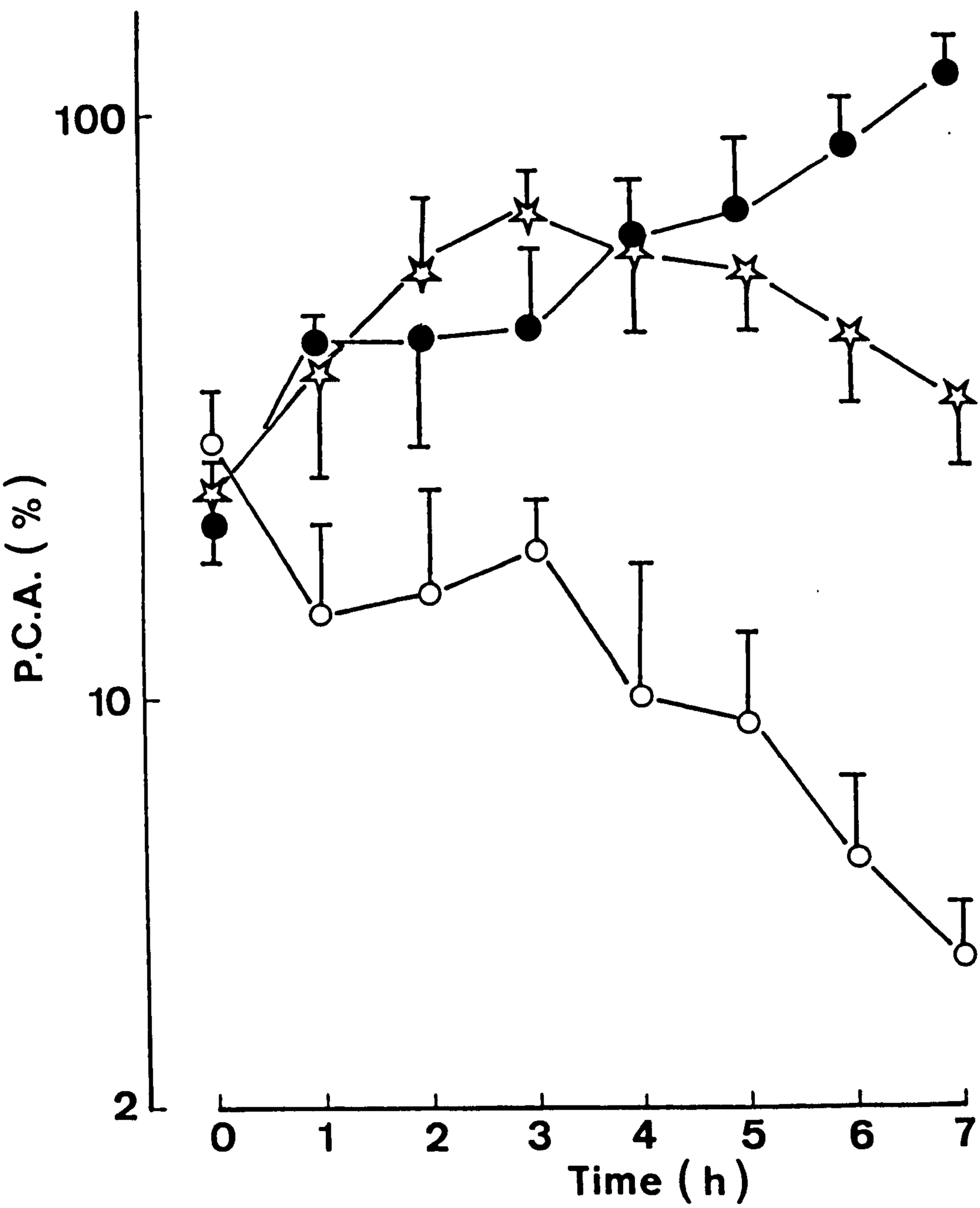


FIGURE 9.4 Prothrombin complex activity (P.C.A.) vs. time in rats following intravenous administration of vitamin K_1 at doses of 10 mg kg^{-1} (●), 1 mg kg^{-1} (☆) and 0.5 mg kg^{-1} (○) 24 h after intraperitoneal administration of brodifacoum (10 mg kg^{-1}).

Results are expressed as means (n = 6) and vertical bars show s.d. mean.



steadily throughout the 7 h period at a rate indicative of maximum inhibition of clotting factor synthesis (2.03 $\pm$ 0.17 h). Conversely P.C.A. reached a maximum (68 $\pm$ 18%) 3 h after administration of a single dose of vitamin K_1 at 1.0 mg kg^{-1}. A single 10 mg kg^{-1} dose of vitamin K_1 produced a steady increase in P.C.A. throughout the 7h period in which the pharmacological response was studied.

Therefore to determine the minimum plasma and hepatic concentrations of vitamin K_1 required to drive clotting factor synthesis, groups of rats received either brodifacoum (10 mg kg^{-1}) or warfarin (63 mg kg^{-1}) followed by a single dose of vitamin K_1 (1.0 mg kg^{-1}). Figure 9.5 illustrates the pharmacological response to vitamin K_1 (1.0 mg kg^{-1}) in those animals from which terminal blood and liver samples were obtained 3 h after administration of vitamin K_1. Following administration of vitamin K_1, plasma concentrations of the vitamin were significantly ($P < 0.02$) reduced in both the warfarin (185 $\pm$ 95 ng ml^{-1}) and brodifacoum (194 $\pm$ 110 ng ml^{-1}) pretreated animals when compared to controls (813 $\pm$ 486 ng ml^{-1}). Vitamin K_1 2,3-epoxide was detected in the plasma of both warfarin (215 $\pm$ 79 ng ml^{-1}) and brodifacoum (263 $\pm$ 78 ng ml^{-1}) pretreated animals, however vitamin K_1 2,3-epoxide was detected in only one of the six control animals (122 ng ml^{-1}) (Figure 9.6).

Similarly hepatic vitamin K_1 concentrations were significantly ($P < 0.001$) reduced by both warfarin (2.95 $\pm$ 1.70 µg g^{-1}) and brodifacoum (2.93 $\pm$ 1.18µg g^{-1}) compared with controls (18.47 $\pm$ 1.08 µg g^{-1}); there was no difference between warfarin and brodifacoum pretreated animals (Figure 9.7). However, vitamin K_1 2,3-epoxide concentrations were significantly ($P < 0.01$) raised by both warfarin (4.43 $\pm$ 2.00 µg g^{-1}) and brodifacoum (4.28 $\pm$ 1.75 µg g^{-1}) when compared with controls

FIGURE 9.5 Prothrombin complex activity (P.C.A.) in rats up to 3h following intravenous administration of vitamin K_1 (1 mg kg^{-1}) 24h after intraperitoneal administration of 0.9% w/v NaCl solution (●), 10 mg kg^{-1} brodifacoum (☆) or 63 mg kg^{-1} warfarin (★). Results are expressed as means (n = 6) and vertical bars show s.e. mean.

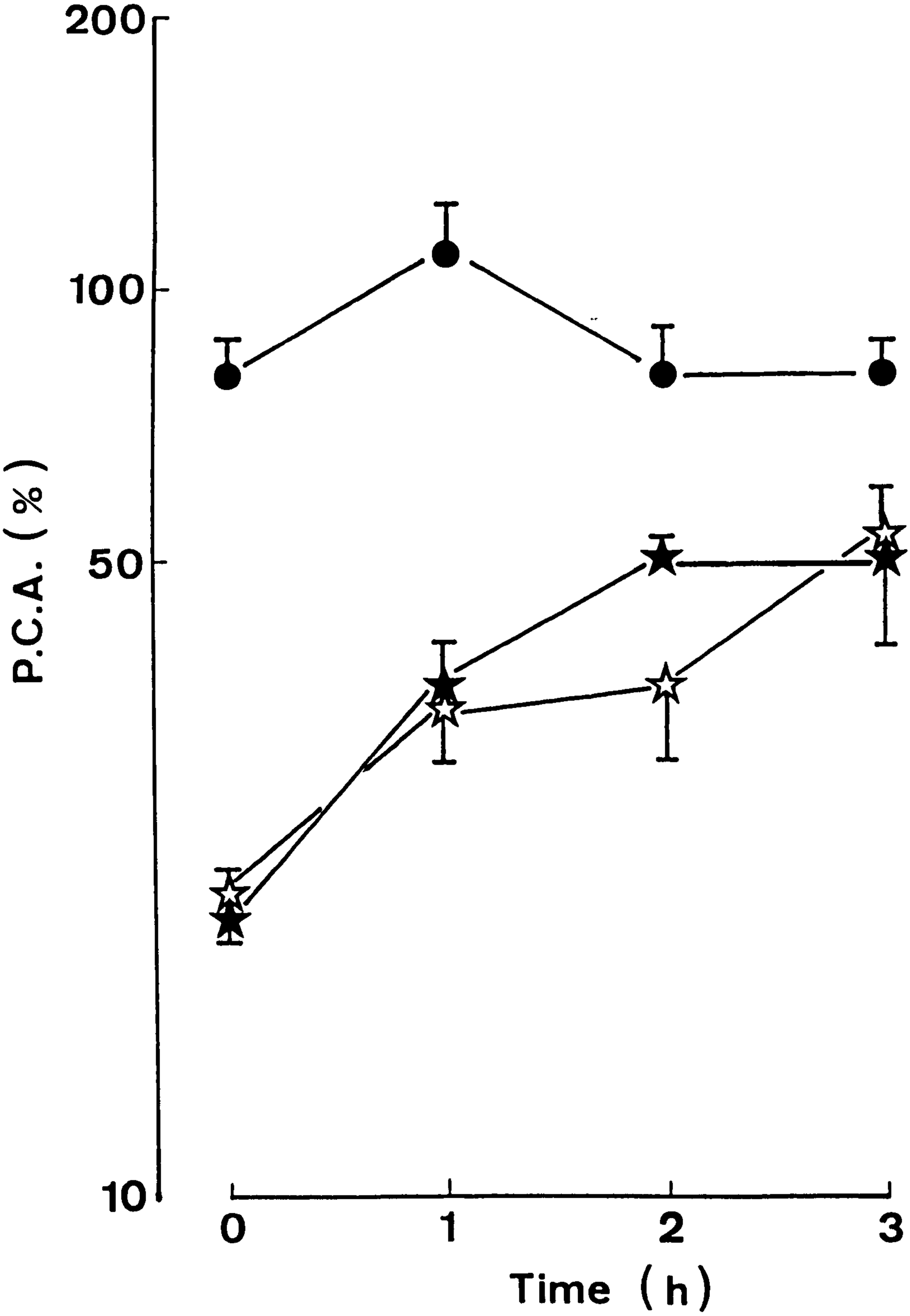


FIGURE 9.6 Plasma concentrations of vitamin K_1 (□) and vitamin K_1 2,3-epoxide (▨) 3h after intravenous administration of vitamin K_1 (1 mg kg^{-1}) to control (C), brodifacoum (B) and warfarin (W) pretreated rats.
Results are expressed as means (n = 6) and vertical bars show s.e. mean.
ND - non detected
★ significantly different from control group at $P < 0.02$.

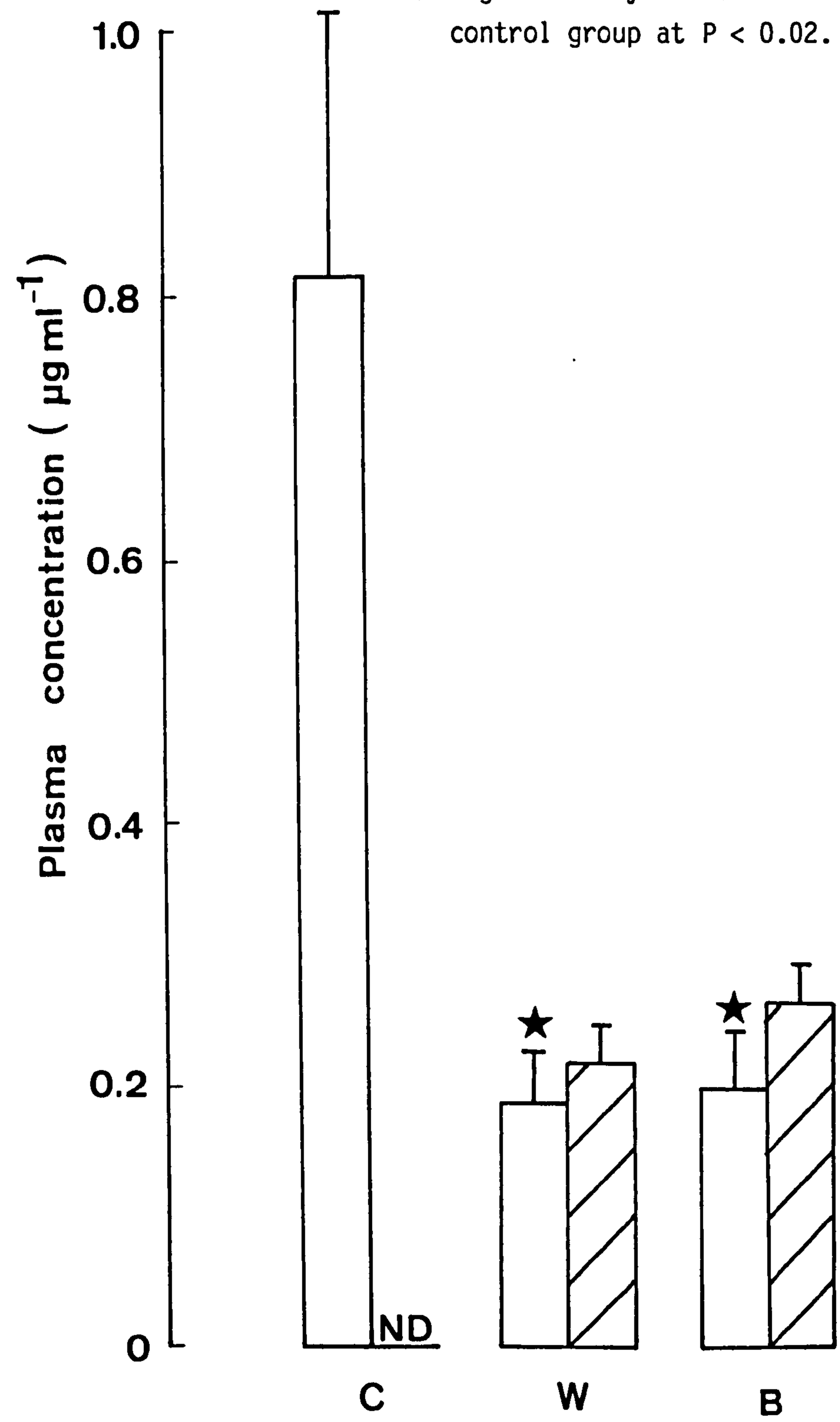
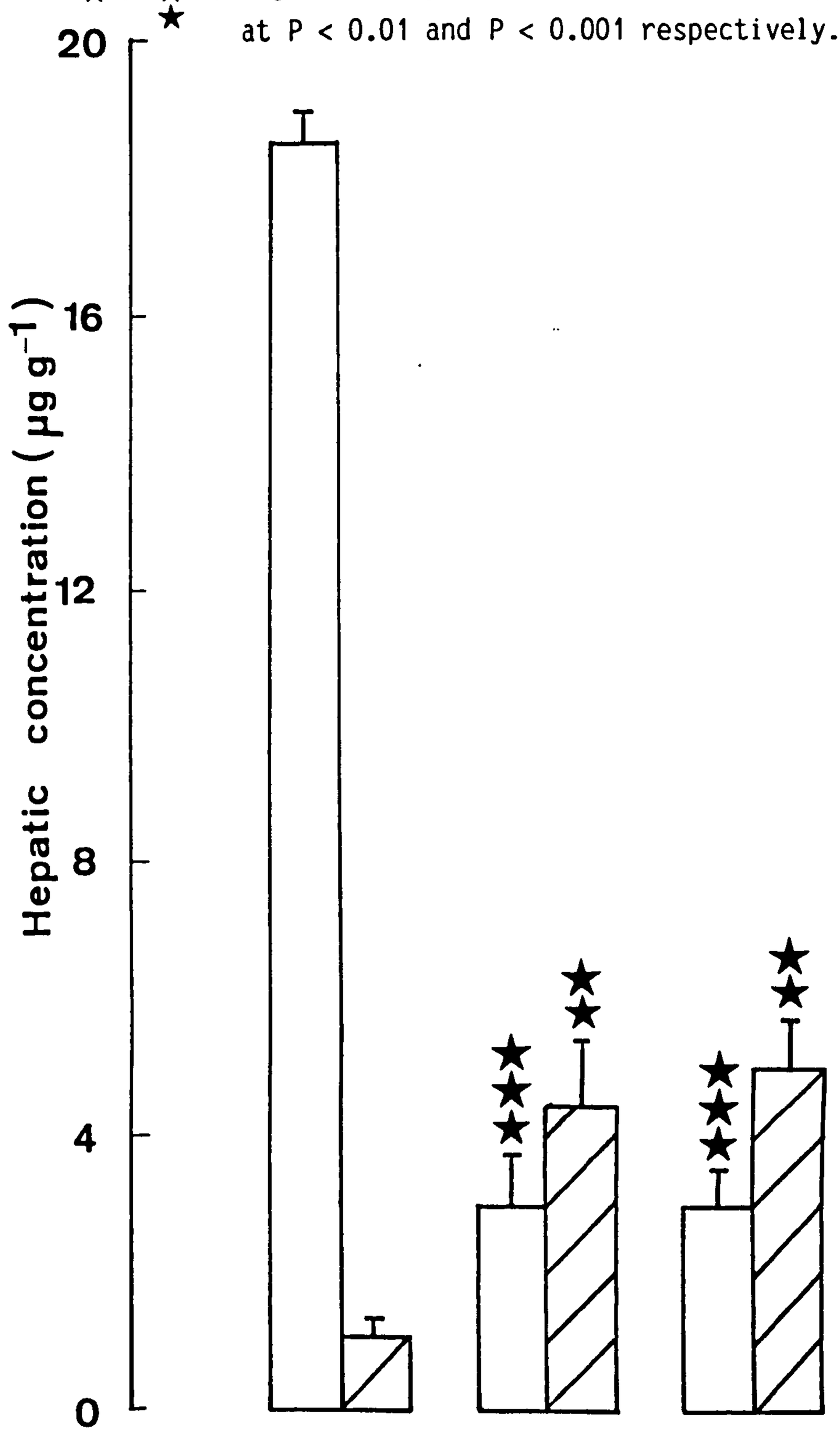


FIGURE 9.7 Hepatic concentrations of vitamin K_1 (□) and vitamin K_1 2,3-epoxide (▨) 3h after intravenous administration of vitamin K_1 (1 mg kg^{-1}) to control (C), brodifacoum (B) and warfarin (W) pretreated rats.
Results are expressed as means (n = 6) and vertical bars show s.e. mean.
★, ★★, ★★★, significantly different from control group at $P < 0.01$ and $P < 0.001$ respectively.



(1.06 ± 0.48 μg g^{-1}); there was no significant difference between warfarin and brodifacoum pretreated animals.

There was no significant difference in the ratio of *cis*: *trans* vitamin K_1 between warfarin and brodifacoum treated animals (Table 9.1). However the difference in the ratio of the isomers between both anti-coagulated groups and the control group was significant ($P < 0.001$).

TABLE 9.1 Hepatic *cis*: *trans* vitamin K_1 ratio in anticoagulated rats.

Pretreatment	Dose (mg kg^{-1})	*cis*: *trans* vitamin K_1 ratio
Control		0.30 ± 0.08
Warfarin	63	2.00 ± 0.30*
Brodifacoum	10	1.75 ± 0.26*

Values show mean ± standard deviation of mean (n = 4)

* Significantly different from control group, $P < 0.001$

9.5 Discussion

Vitamin K_1 is the antidote most frequently used in the treatment of coumarin overdosage, therapeutic or deliberate (O'Reilly and Aggeler, 1976). With the development of new rodenticidal coumarin anticoagulants such as brodifacoum and difenacoum, which are more potent and persistent vitamin K_1 antagonists than warfarin (Park and Leck, 1982), the short duration of action of vitamin K_1 during coumarin overdose has become more evident in both man (Barlow et al., 1982; Jones et al., 1984a; Lipton and Klass, 1984) and experimental animals (Hart et al., 1984; Park et al., 1984). Current therapeutic advice is that intravenous administration of the vitamin is used initially, but an oral formulation may be used later (British National Formulary). Administration of the vitamin is continued ad hoc until the prothrombin time returns to normal.

Thus the aim of the present study was to develop an animal model which would mimic the situation of chronic coumarin anticoagulation in man. The rat was chosen for many reasons (section 9.2) but mainly because nearly all the previous work concerned with the hepatic disposition of vitamin K_1 has been conducted in this species. Much of this work has shown that vitamin K_1 undergoes similar metabolism in both man (Park et al., 1984) and the rat (Leck and Park, 1981).

Although several studies have investigated the effect of chronic coumarin anticoagulation on the disposition of vitamin K_1, these studies have generally been limited to plasma concentration data (Park et al., 1979; Hart et al., 1984). However by using the rat as an animal model, it is possible not only to compare the plasma concentrations of vitamin K_1 with pharmacological response to the

vitamin, but also to determine the hepatic concentration of vitamin K_1 required to drive clotting factor synthesis in the anticoagulated rat.

The pharmacodynamic response to vitamin K_1 in the chronically anticoagulated animal was determined by the measurement of P.C.A. Doses of warfarin and brodifacoum were chosen which would produce maximum antagonism of vitamin K_1 for at least 24 hours. To produce comparable antagonism to that of brodifacoum, chronic administration of ten molar equivalents of warfarin was required. According to current concepts, this represents the limiting situation in which the physiologically important, microsomal enzyme vitamin K epoxide reductase is non-functional. In agreement with this notion, recent work has shown that both warfarin and brodifacoum can completely inhibit the *in vivo* reduction of vitamin K_1 2,3-epoxide (B.P. Haynes, Ph.D. Thesis 1987). Brodifacoum is therefore a useful research tool for the investigation of vitamin K_1 requirements in animals which lack a functional vitamin K epoxide reductase.

The pharmacological response to vitamin K_1 (1 mg kg^{-1}) was identical in both warfarin (63 mg kg^{-1}) and brodifacoum (10 mg kg^{-1}) anticoagulated animals. P.C.A. reached a maximum 3 h after administration of vitamin K_1 and then declined at a rate indicative of complete inhibition of clotting factor synthesis. As the duration of action of vitamin K_1 terminates sharply at 3 h, this provides a sharp pharmacological end point, and is in accordance with previous studies in chronically coumarin anticoagulated conscious rabbits (Park *et al.*, 1984). However, in the present study vitamin K_1 (0.5 mg kg^{-1}) was ineffective in restoring the synthesis of clotting factors in brodifacoum anticoagulated rats. Vitamin K_1 (10 mg kg^{-1})

produced a continuous increase in P.C.A. throughout the 7 h period following its administration to brodifacoum anticoagulated rats. There was a very sharp initial rise in P.C.A. during the first hour, this probably reflects rapid γ-carboxylation of clotting factor precursors which have accumulated in the liver (Suttie, 1970). The inability of coumarin anticoagulants to completely antagonise vitamin K_1 is consistent with the hypothesis that they act indirectly by inhibition of vitamin K epoxide reductase and do not block γ-carboxylation directly (Bell, 1978; Park et al., 1979; Fasco et al., 1983).

Measurement of the plasma concentration of vitamin K_1 and vitamin K_1 2,3-epoxide 3 h after vitamin K_1 administration show that both warfarin and brodifacoum significantly reduce the concentration of vitamin K_1 when compared to control animals. This finding is in agreement with the rapid clearance of vitamin K_1 in patients poisoned with coumarin anticoagulants. However, it has been shown previously that coumarin anticoagulants have no effect on the rate of elimination of vitamin K_1 from plasma in either man (Shearer et al., 1977) or the rabbit (Park et al., 1979). However, 4-hydroxycoumarins have been shown to cause an increase in the amount of vitamin K_1 2,3-epoxide in the plasma of man (Shearer et al., 1977) and the rabbit (Park et al., 1979). This is consistent with the findings of the present study, in which only one of the six control rats had detectable amounts of vitamin K_1 2,3-epoxide in plasma (122 mg ml^{-1}), whereas those rats pretreated with warfarin and brodifacoum had significantly elevated concentrations of the epoxide. After administration of a pharmacological dose of the vitamin, vitamin K_1 2,3-epoxide could not be detected in man in the absence of coumarin anticoagulants (Park et al., 1984). However, rabbits display relatively high concentrations of

vitamin K_1 2,3-epoxide ($\sim$ 5 $\mu g\ ml^{-1}$) following vitamin K_1 (10 $mg\ kg^{-1}$) (Hart et al., 1984).

In the present study, 3 h after administration of a pharmacological dose of vitamin K_1, the hepatic concentrations of vitamin K_1 in the groups of rats anticoagulated with warfarin and brodifacoum were dramatically reduced. Concentrations were less than 20% of those in controls, while the ratio of *trans*-vitamin K_1 (the active form of the vitamin) to *cis* - vitamin K_1 (the inactive form of the vitamin) had increased from 0.30 ± 0.08 in control rats to 2.00 ± 0.30 in warfarin and 1.75 ± 0.26 in brodifacoum treated rats. Thus during chronic coumarin anticoagulation, approximately two thirds of the vitamin present in the liver is in the form of *cis* - vitamin K_1. This can probably be explained by the fact that as the active *trans* - vitamin K_1 is used up in the vitamin K - epoxide cycle, the relative proportion of *cis* - vitamin K_1 increases as this form cannot be utilized in clotting factor synthesis (Knauer et al., 1975).

Those rats which were pretreated with either warfarin or brodifacoum showed a significant increase in the hepatic concentration of vitamin K_1 2,3-epoxide when compared to controls. This is consistent with previous observations in coumarin anticoagulated rats (Caldwell et al., 1974; Leck and Park, 1981). The total amount of vitamin K_1 and vitamin K_1 2,3-epoxide in the warfarin and brodifacoum pretreated groups amounted to less than half that determined in the control group. This can probably be explained by the fact that in the situation where the enzyme vitamin K epoxide reductase is non-functional, vitamin K_1 is converted to vitamin K_1 2,3-epoxide and as the epoxide cannot be recycled to vitamin K_1 it is metabolised and excreted as metabolites of vitamin K_1 2,3-epoxide. The phenomenon of coumarin poisoning

being associated with a much reduced capacity to conserve vitamin K_1, may in part explain why such large, repeated doses of vitamin K_1 are required to maintain clotting factor synthesis in this situation.

The plan of this study allowed the minimum plasma and hepatic concentrations of vitamin K_1 required to drive clotting factor synthesis to be determined in the rat. The plasma concentration obtained was 0.06 to 3.25 $\mu g\ ml^{-1}$ which compares to that obtained in brodifacoum anticoagulated rabbits (0.4 to 1.0 $\mu g\ ml^{-1}$: Hart et al., 1984). However, the main aim of this study was to determine the concentration of vitamin K_1 at the site of clotting factor synthesis. The high hepatic concentration (1.2 to 6.3 $\mu g\ g^{-1}$) required to drive clotting factor synthesis in chronically coumarin anticoagulated rats contrasts markedly with normal hepatic concentrations of the vitamin in the same species (8 $ng\ g^{-1}$: Haroon and Hauschka, 1983), and the estimated body pool of vitamin K_1 ca 100 $\mu g\ kg^{-1}$ (Duello and Matschiner 1972); Bjornsson et al., 1979). However, the small pool of vitamin K_1 associated with clotting factor synthesis is normally conserved in the vitamin K - epoxide cycle and may contain high molecular weight forms of the vitamin (menaquinones -7, -9, and -10) which are more active on a molecular weight basis than vitamin K_1 (Wiss and Gloor, 1966; Matschiner and Taggart, 1968).

Alternatively the lack of ability of the liver to replenish the pool of vitamin K_1, once it has been depleted by coumarin anticoagulation, may be due to a slow rate of interchange between exogenous vitamin K_1 and physiological vitamin K_1 at its site of action. The metabolism of vitamin K_1 includes the cyclic reduction of vitamin K_1 quinone to the hydroquinone oxidation to the epoxide and its reduction to vitamin K_1 quinone. All enzyme activities catalysing these reactions, vitamin

K-dependent carboxylase, epoxidase, epoxide reductase and vitamin K reductase have been located in the rough endoplasmic reticulum in rat liver (Carlise and Suttie, 1980). In the normal situation the vitamin K_1 utilized by these enzymes is located in the body pool of the vitamin and is sufficient to maintain clotting factor synthesis. However, in the limiting situation of chronic coumarin anticoagulation, produced experimentally in the present study and displayed in patients poisoned with coumarin anticoagulants, the physiological pool of vitamin K_1 will be used up relatively quickly, as the enzyme responsible for the recycling of vitamin K_1, vitamin K_1-epoxide reductase has been rendered non-functional. If interchange between the two sources of vitamin K_1 is slow, due to difficulty in the highly lipophillic vitamin K_1 reaching the enzymes deeply embedded in the rough endoplasmic reticulum, then clotting factor synthesis will ultimately cease.

9.6 Conclusions

The present study has shown that the rat is a useful animal model with which to determine the relationship between hepatic concentrations and pharmacological effect of vitamin K_1, during coumarin anticoagulation. Using this experimental system, the hepatic concentration of vitamin K_1 required to drive clotting factor synthesis in the presence of a non-functional vitamin K epoxide reductase was found to be approximately 100 times greater than that present under normal circumstances.

The utilisation of the active *trans* - vitamin K_1 in clotting factor synthesis, resulted in a relative increase in the ratio of *cis* : *trans* vitamin K_1 during coumarin anticoagulation.

Although there is limited use in monitoring hepatic concentrations of vitamin K_1, as this cannot be carried out in man, the only way in which more information concerned with the disposition of vitamin K_1 in chronic coumarin anticoagulation can be determined is by developing animal models which as near as possible mimic the situation of coumarin poisoning, and also by studying individual cases of coumarin poisoning in man.

CHAPTER 10

CONCLUDING DISCUSSION

Since the successful clinical trials of the early 1940s, 4-hydroxycoumarin anticoagulants have been the major group of pharmacological agents used in the treatment and prophylaxis of thromboembolic and vascular disorders. As a consequence of many studies in man and experimental animals, and *in vitro* studies using enzyme preparations, our knowledge of the pharmacology of these compounds and their antagonism of vitamin K_1 has improved considerably over the past 40 years.

The role of vitamin K as an essential co-factor for the post-ribosomal γ-carboxylation of glutamic acid residues in precursor proteins, resulting in the production of active clotting factors II, VII, IX and X is well established (Jackson and Suttie, 1977; Stenflo and Suttie, 1977). During this process vitamin K_1 is known to be converted to vitamin K_1 2,3-epoxide by the enzyme vitamin K epoxidase. Vitamin K-dependent carboxylase and epoxidase enzyme activities are thought to be, in someway, coupled. For continued clotting factor synthesis, the inactive vitamin K_1 2,3-epoxide is converted back to vitamin K_1 by the enzyme vitamin K epoxide reductase. This recycling mechanism is known as the vitamin K epoxide cycle (Willingham and Matschiner, 1974; Bell, 1978) and the vitamin K_1 utilized in this biochemical system is thought to be associated with the body pool of vitamin K. It is now thought that vitamin K_1 must be converted to vitamin K_1 hydroquinone before it can be used as a co-factor in the carboxylation of glutamic acid residues in clotting factor precursors. The enzyme responsible for this conversion is vitamin K reductase (Wallin and Suttie, 1981; Whitlon *et al.*, 1978), and recent evidence suggests that the reduction of vitamin K_1 quinone and vitamin K_1 2,3-epoxide is catalysed by either a single enzyme, or a multisite enzyme complex (Fasco and Principe,

1980; Hildebrandt and Suttie, 1982). It is now widely accepted that coumarin anticoagulants such as warfarin, exert their pharmacological effect by inhibition of the vitamin K / vitamin K epoxide reductase enzyme system, and as a consequence, prevent the regeneration of vitamin K in the vitamin K-epoxide cycle. This leads to inhibition of the synthesis of the vitamin K-dependent clotting factors, resulting in a delayed anticoagulant effect dependent on the rate of clotting factor degradation. As a consequence of their indirect mode of action, the plasma concentrations of coumarin anticoagulants do not reflect their pharmacological effect.

One of the major problems associated with the use of coumarin anticoagulants is the inter-individual variation in pharmacological response to a standard dose (Breckenridge, 1977). This can be due to differences in both the pharmacokinetics and the pharmacodynamics of the drug. Thus to understand further the pharmacology of coumarin anticoagulants, it is important to be able to distinguish between the pharmacokinetic and the pharmacodynamic processes. However, the lack of a direct relationship between the plasma concentration and the pharmacological effect of these compounds, made this distinction difficult. To overcome this problem, Nagashima and co-workers (1969) developed the concept of rate of synthesis of prothrombin complex activity (R_{syn}), which describes the extent and time course of changes in clotting factor concentrations, and was shown to be directly related to the log of the plasma concentration of warfarin.

In the present work (Chapter 3), an animal model was developed to determine the relative contributions of the pharmacokinetic and the pharmacodynamics of any 4-hydroxycoumarin anticoagulant to the pharmacological effect of that drug observed <u>in vivo</u>. The rabbit was chosen

as the animal model because the pharmacokinetics and the pharmacodynamics of the coumarin anticoagulant could be determined simultaneously in the same animal. The model was used to investigate the pharmacology of racemic warfarin and its enantiomers. Following the intravenous administration of single doses of the three forms of warfarin, plasma concentrations were seen to decline mono-exponentially, indicating one compartment pharmacokinetics for warfarin in the rabbit. The pharmacokinetics of racemic warfarin were shown to be independent of dose over a ten-fold dose range, which is consistent with observations in man (O'Reilly et al., 1971; Hackett et al., 1985).

Using the approach, developed by Nagashima and co-workers (1969), mono-phasic recovery of R_{syn} was seen following the three forms of warfarin investigated, and this was dose-dependent for racemic warfarin over a ten-fold dose range. R_{syn} recovered more rapidly after S(-) warfarin than after R(+) and racemic warfarin.

The duration of pharmacological response to a drug is a function of both receptor interaction and elimination from the body. In an attempt to distinguish these two processes, m, the slope of the intensity of effect - log (amount of drug in the body) curve was determined for warfarin and its enantiomers. The value of m, calculated for racemic warfarin, was independent of dose over a ten-fold dose range and was similar to that obtained for R(+) and S(-) warfarin. This indicates that the three forms of warfarin act at the same receptor, thought to be the vitamin K / vitamin K epoxide reductase enzyme system. By making the fundamental assumption that there is simple equilibrium of warfarin and its enantiomers between plasma and hepatic receptor sites, plasma concentration and R_{syn} data was used to determine the minimum concentration required to maximally inhibit clotting

factor synthesis and thus determine the potencies of warfarin and its enantiomers. Despite its shorter half-life, S(-) warfarin was shown to be more potent than R(+) warfarin. However, the relationship between plasma and hepatic concentrations of warfarin has been shown to be more complex than that of simple equilibrium (Covell et al., 1983), thus minimum hepatic concentrations of coumarin anticoagulant required to maximally inhibit clotting factor synthesis would provide a more accurate indication of the potencies of these compounds. Furthermore, the simultaneous determination of hepatic and plasma concentrations of coumarin anticoagulant would more clearly define any limitations of the pharmacokinetic - pharmacodynamic model developed in the present work.

Anticoagulant control in patients is difficult, since it is often complicated by marked intra-individual variation in response to therapy during a single course of treatment. This may be due to the development of a diseased state, or to the introduction of another drug into the patients therapeutic regimen. These factors can significantly alter the pharmacological response to the anticoagulant, and as a consequence of the relatively narrow therapeutic window of these compounds, even a small change in pharmacological effect may place the patient in a life threatening situation. The seriousness of these interactions with coumarin anticoagulants, stress the importance of the determination of the mechanisms by which a change in pharmacological response may be brought about.

The suitability of the mathematical models employed to determine the relative contributions of the pharmacokinetics and the pharmacodynamics of warfarin to its pharmacological effect in vivo in the rabbit, illustrated the potential usefulness of this approach to

determine the mechanism by which thyrotoxicosis potentiates the hypoprothrombinaemic effect of warfarin in man.

In both the hypermetabolic and the euthyroid states, the plasma half-lives of warfarin were similar to those previously documented (O'Reilly and Aggeler, 1968; O'Reilly, 1982), and there was no significant difference in any of the pharmacokinetic parameters of warfarin between the two states. An elevated pharmacodynamic effect as determined by PTR was demonstrated in all patients in the hypermetabolic state. A greater decrease in the activity of factor VII following warfarin administration and a lower basal activity of factor II was observed in the thyrotoxic state when compared to the normal situation.

The production of _in vivo_ concentration - response curves revealed an apparent increased receptor sensitivity to warfarin in the hypermetabolic state. Considering the general physiological effects of the thyroid hormones T_3 and T_4, it is probable that increased sensitivity to warfarin in thyrotoxicosis is due to increased protein (clotting factor) turnover. This theory is supported by the effects of the diseased state on the activity of factors II and VII observed in the study described in this thesis, and also by the results of previous studies, which have shown an increased rate of degradation of factor II in dogs receiving D-thyroxine (Weintraub _et al._, 1973) and an increased rate of degradation of factor II in hyperthyroidism in man (Loeliger _et al._, 1964). Thus, by making the distinction between the pharmacokinetics and the pharmacodynamics of warfarin, a pharmacodynamic effect has been shown to be responsible for the increased effectiveness of warfarin in thyrotoxicosis. To identify the mechanisms responsible for the pharmacodynamic effect, a more detailed study of the individual clotting factors would have to be undertaken.

The clinically significant interaction between warfarin and the H_2 antagonist, cimetidine, is well documented. A study by Serlin and co-workers (1979) indicated that the interaction has a pharmacokinetic basis, and since cimetidine is a well known inhibitor of drug metabolism (Somogyi and Gugler, 1982), it was postulated that the change in the pharmacokinetics of warfarin, when taken with cimetidine, was due to an altered metabolism of the anticoagulant. As a consequence of its complex, stereoselective, metabolic profile, warfarin is a useful drug to use for the investigation of inhibition and induction of the isozymes of cytochrome P_{450}.

In the study described in this thesis, a significant change in the pharmacokinetics of R(+) warfarin was observed in the presence of cimetidine, but there was no change in the pharmacokinetics of S(-) warfarin. Consideration of the different metabolic profiles of the enantiomers of warfarin, led to the conclusion that 6-hydroxylation, upon which R(+) warfarin relies heavily for its metabolism in man, but which is only a minor pathway for the metabolism of S(-) warfarin, is inhibited by cimetidine. Consistent with the present concept of multiple forms of cytochrome P_{450}, each of which may be responsible for different metabolic pathways and also susceptible to inhibition and induction by different agents, it appears that a particular form(s) is susceptible to selective inhibition by cimetidine.

In conclusion, this study illustrates the usefulness of plasma concentration data for the determination of the mechanisms responsible for pharmacokinetic interactions with warfarin. Furthermore, the results are in direct contrast to those determined for stereoselective interactions of warfarin with other drugs. These include metronidazole (O'Reilly, 1976) and phenylbutazone (Banfield et al., 1983), which

have been shown to cause an increase in the plasma half-life of S(-) warfarin, whereas the study described in this thesis, has shown an interaction with the R enantiomer of warfarin. This questions the suggestion, by O'Reilly (1976), that the clinical use of R(+) warfarin instead of racemic warfarin would prevent drug interactions.

In patients on well controlled coumarin anticoagulant therapy, a fine balance is achieved between under-anticoagulation where the individual is at risk of thrombus formation, and over anticoagulation which brings with it the risk of haemorrhage. Consequently, patients taking oral anticoagulants require regular measurement of prothrombin time. In Chapter 6, daily dose of warfarin and the plasma concentrations of racemic and S(-) warfarin were investigated as predictors of pharmacological effect in a large number of patients on steady state warfarin therapy. These parameters were shown to be poor predictors of anticoagulant effect, which suggests that inter-individual differences in receptor sensitivity are important to determinants of the inter-individual variation in pharmacological effect. Plasma concentrations of vitamin K_1 2,3-epoxide, following a pharmacological dose of the vitamin, were therefore investigated as a possible marker of warfarin action. Vitamin K_1 2,3-epoxide was detected in the plasma of a small group of patients on steady state warfarin therapy. This is consistent with the proposed mechanism of action of coumarin anticoagulants, the inhibition of the enzyme vitamin K epoxide reductase, and illustrates that the presence of vitamin K_1 2,3-epoxide in plasma is a useful marker for mechanistic studies. However, it was shown to be a poor quantitative predictor of pharmacological effect, illustrating that it is perhaps inter-individual differences in protein (clotting factor) metabolism rather than differences in vitamin K_1 meta-

bolism which account for the inter-individual variation in pharmacological response to warfarin.

In Chapter 7, vitamin K_1 2,3-epoxide was investigated as a marker of warfarin action in controlled volunteer studies. Following a pharmacological dose of vitamin K_1, vitamin K_1 2,3-epoxide was detected in the plasma of volunteers on daily doses of warfarin much lower than those commonly administered therapeutically. By combining the results obtained in Chapters 6 and 7, concentration-response (plasma concentration of vitamin K_1 2,3-epoxide) curves were produced which suggest concentration-dependent inhibition of vitamin K epoxide reductase. This has previously been demonstrated following tracer doses of vitamin K_1 during anticoagulation with single doses of warfarin (Shearer et al., 1977).

The detection of vitamin K_1 2,3-epoxide in plasma, was shown to be a more sensitive indicator of warfarin presence than the commonly used prothrombin time. Measurement of γ-carboxylated prothrombin (Blanchard et al., 1983) by radioimmunoassay, has been shown to be approximately 1000 times more sensitive than prothrombin time as a measure of changes in clotting factor activity. It would be of interest, therefore, to determine the relationships between plasma warfarin, vitamin K_1 2,3-epoxide accumulation and prothrombin antigen.

S(-) warfarin is known to be more potent than R(+) warfarin in man (Breckenridge et al., 1974; Wingard et al., 1978). Since there is considerable evidence to suggest that the warfarin receptor is associated with the enzyme vitamin K epoxide reductase, plasma concentrations of vitamin K_1 2,3-epoxide were investigated as a marker of coumarin anticoagulant action, to determine whether a stereoselective effect

exists at the receptor. Previous studies in man have failed to reveal any difference (Shearer et al., 1977; Choonara et al., 1986), possibly because the doses of warfarin employed exceeded that required for the maximum accumulation of vitamin K_1 2,3-epoxide. However, the production of concentration - response (plasma concentration of vitamin K_1 2,3-epoxide) curves from the results obtained in the present work, revealed a greater potency of S(-) warfarin at 1 mg daily when compared to R(+) warfarin at the same daily dose. Thus plasma concentration of vitamin K_1 2,3-epoxide, following a pharmacological dose of vitamin K_1, has been shown to be a useful marker of warfarin action in well defined, controlled volunteer studies. Furthermore, this parameter appears to be more sensitive to the effects of warfarin than the commonly used prothrombin time. The importance of this difference in sensitivity was illustrated in a recent study in which workers, previously exposed to the novel coumarin anticoagulants, brodifacoum and difenacoum, showed normal prothrombin times but high plasma concentrations ($\sim$ 300 ng ml^{-1}) of vitamin K_1 2,3-epoxide (Park et al., 1986). This observation questions the use of prothrombin time as the sole determinant of coumarin anticoagulant presence to assess industrial coumarin exposure (accumulation), and illustrates the need for a marker of vitamin K metabolism, such as plasma concentration of vitamin K_1 2,3-epoxide. Furthermore, this would provide a useful marker of coumarin presence in individuals who surreptitiously take these compounds.

Salicylate and some N-methyl-thiotetrazole containing antibiotics have been shown to exert a weak anticoagulant effect in man (Barrow et al., 1967; Schimada et al., 1984). Following a pharmacological dose of vitamin K_1 to individuals taking salicylate, latamoxef or cefamandole, vitamin K_1 2,3-epoxide was detected in plasma (Bechtold,

1983). This illustrates the usefulness of vitamin K_1 2,3-epoxide as a determinant of the mechanism of action of such interactions with vitamin K_1.

Until recently, investigation of the metabolism of vitamin K_1 required administration of either a radiolabelled, or a pharmacological dose of the vitamin to enable measurement of vitamin K_1 in biological material. In the studies described in Chapter 8, a modified version of an HPLC method with electrochemical detection (Hart et al., 1985) was employed to determine physiological concentrations of vitamin K_1 in biological material. Using this method, the disposition of a pharmacological dose of vitamin K_1 in relation to normal physiological concentrations of the vitamin was studied in man and the rabbit. Brodifacoum was used as an experimental tool for the maintenance of vitamin K_1 antagonism in the rabbit. At 24 h after treatment with this novel 4-hydroxycoumarin anticoagulant, physiological plasma concentrations of vitamin K_1 were significantly reduced.

In previous studies of the plasma disposition of vitamin K_1, in man and the rabbit, plasma concentrations displayed bi-exponential decay. In the present work, using more sensitive analytical techniques, a third phase of vitamin K_1 elimination was determined in both species. This phase of elimination was shown to continue until plasma concentrations in both man and the control rabbits were similar to normal physiological concentrations, and those in the brodifacoum pretreated rabbits were similar to the sub-normal concentrations determined 24 h after anticoagulant administration.

Although brodifacoum pretreatment resulted in significantly decreased physiological plasma concentrations of vitamin K_1 in the rabbit, the pharmacokinetics of an intravenous, pharmacological dose

of vitamin K_1 were unaffected by brodifacoum pretreatment. This suggests that the elimination of vitamin K_1 from plasma may be dependent on factors such as liver blood flow rather than hepatic metabolism. To investigate this idea further, a system is required in which hepatic blood flow could be altered. The isolated perfused liver system has been successfully used to investigate the metabolism of a number of drugs including propranolol (Jones et al., 1984b) and primaquine (Mihaley et al., 1985). However, during the course of the present work, preliminary experiments have indicated that, as a consequence of its lipophilic nature, at least 70% of a pharmacological dose of vitamin K_1 binds to the polypropylene tubing of this experimental system. Thus an all glass isolated perfused liver system, which could be treated with silylating agent to prevent binding of the vitamin to the glass, would have to be used.

Previous studies in man and experimental animals have illustrated that very high plasma concentrations of vitamin K_1 are required to drive clotting factor synthesis during chronic coumarin anticoagulation (Barlow et al., 1982; Hart et al., 1984). In the present work, in brodifacoum pretreated rabbits, P.C.A. recovered rapidly immediately following the administration of a pharmacological dose of vitamin K_1, at which time the plasma concentrations of vitamin K_1 were 10,000 times greater than normal physiological concentrations. However, 2 to 6 h following vitamin K_1 administration, P.C.A. began to decline, even though plasma concentrations of the vitamin were between 1000 and 2500 times greater than physiological concentrations. Thus it appears that below a certain plasma concentration, vitamin K_1 cannot be readily incorporated into the body pool of vitamin K_1 and therefore cannot be utilized to drive clotting factor synthesis. These results illustrate

that plasma concentrations of vitamin K_1 do not reflect the concentration of the vitamin at the site of clotting factor synthesis in the liver. Consistent with this hypothesis, hepatic concentrations of vitamin K_1 in the rabbit were depleted by pretreatment with brodifacoum. Thus, in the case of the coumarin anticoagulated rabbit, physiological concentrations of vitamin K_1 in plasma reflect the concentrations at the site of clotting factor synthesis, but the pharmacokinetics of a pharmacological dose of the vitamin, do not. These results indicate the importance of the determination of hepatic concentrations of vitamin K_1. In Chapter 9 an animal model was developed for this purpose. The rat was chosen for this model because (a) many animals have to be sacrificed to obtain few data points, and (b) a considerable amount of information concerned with vitamin K_1 and coumarin anticoagulants have been obtained using this species (Ren et al., 1977; Leck and Park, 1981).

The duration of action of a 1.0 mg kg^{-1} dose of vitamin K_1 terminated sharply 3 h after administration to brodifacoum and warfarin anticoagulated animals. This provided a sharp pharmacological end point at which time hepatic concentrations of vitamin K_1 and vitamin K_1 2,3-epoxide were investigated. In both brodifacoum and warfarin anticoagulated animals, hepatic concentrations of vitamin K_1 were significantly lower than those in control animals, and hepatic concentrations of vitamin K_1 2,3-epoxide were significantly higher than those in control rats. The combined hepatic concentrations of vitamin K_1 and vitamin K_1 2,3-epoxide in the anticoagulated animals was less than that in control animals. This suggests that vitamin K_1 from a pharmacological dose reaches the site of clotting factor synthesis and is subsequently converted to vitamin K_1 2,3-epoxide. This

cannot be recycled back to vitamin K_1 due to inhibition of vitamin K epoxide reductase by either brodifacoum or warfarin, and is therefore metabolised and excreted as metabolites of vitamin K_1 2,3-epoxide. Alternatively, for some reason, vitamin K_1 from a pharmacological dose may not be able to reach the site of clotting factor synthesis during coumarin anticoagulation.

Consistent with the hypothesis that *cis*-vitamin K_1 is inactive in clotting factor synthesis (Knauer et al., 1975), coumarin anticoagulation with either brodifacoum or warfarin resulted in a relative increase in the ratio of *cis* : *trans*-vitamin K_1 in the liver. This indicates that *trans*-vitamin K_1 is utilized to drive clotting factor synthesis.

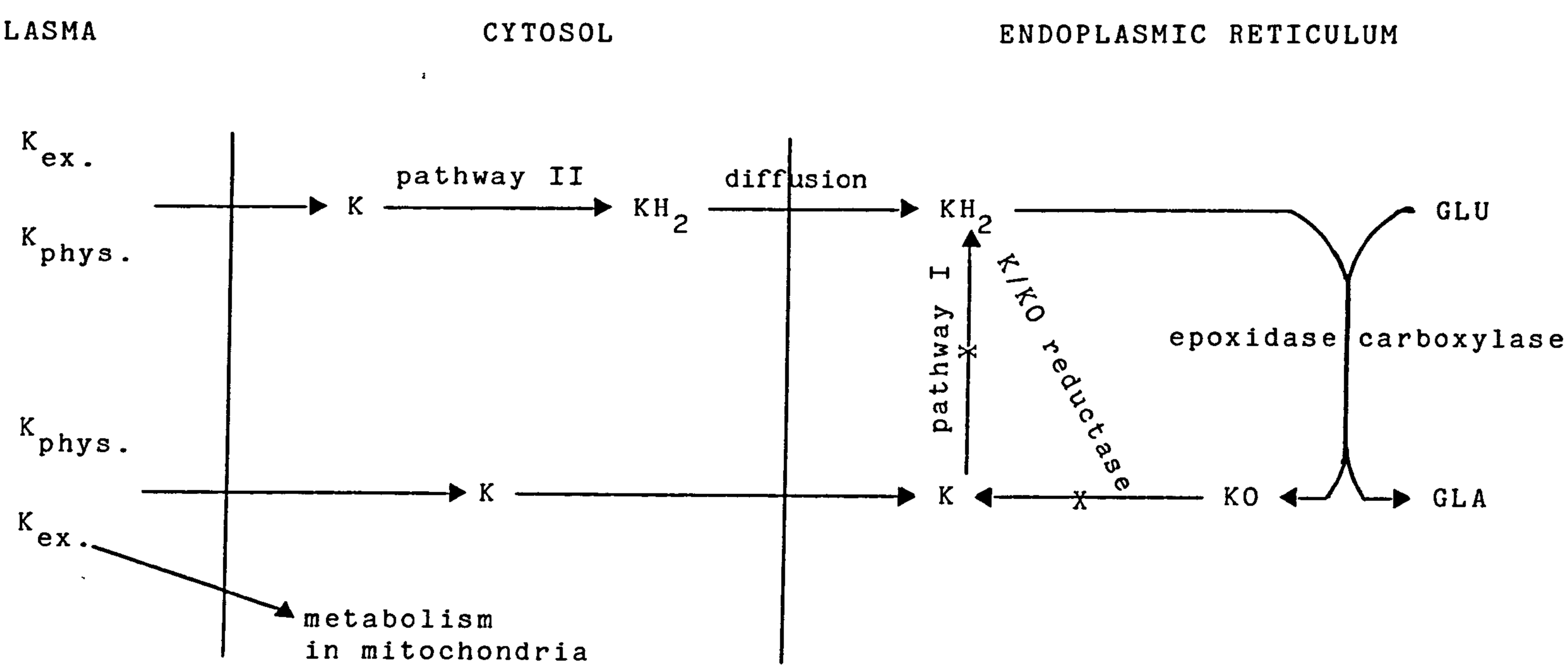
Under normal circumstances the vitamin K - epoxide cycle appears to regenerate vitamin K_1 from the inactive metabolite, vitamin K_1 2,3-epoxide, and therefore conserve the body pool of the vitamin which is vital for blood coagulation mechanisms to function normally. However, during anticoagulation with the coumarin anticoagulants, warfarin and brodifacoum, vitamin K_1 2,3-epoxide was detected in the plasma of both man and experimental animals in the studies described in this thesis. This is consistent with the hypothesis that coumarin anticoagulants exert their pharmacological effect by inhibition of the enzyme vitamin K epoxide reductase (Bell, 1978). This enzyme is very similar to a microsomal reductase enzyme which catalyses the production of vitamin K hydroquinone, which is essential for the γ-carboxylation of glu-residues in clotting factor precursors. This vitamin K reductase, like vitamin K epoxide reductase, requires a sulphydryl compound as a co-factor for its activity (dithiothreitol (DTT) in vitro; Fasco and Principe, 1980) and is susceptible to inhibition by coumarin anticoag-

ulants (Hildebrandt and Suttie, 1982). These similarities have contributed to the concept that vitamin K and vitamin K epoxide reductase are in fact, either a single enzyme or a multisite enzyme complex.

The conversion of vitamin K to vitamin K hydroquinone is also catalysed by the pyridine nucleotide-dependent dehydrogenase enzyme, DT-diaphorase which occurs primarily in the cytoplasm of hepatocytes (Wallin and Suttie, 1981). This vitamin K reductase is relatively insensitive to inhibition by coumarin anticoagulants (Fasco and Principe, 1982). The pathway catalysed by the warfarin sensitive microsomal reductase (Pathway I) is thought to be primarily responsible for the generation of vitamin K hydroquinone *in vivo*. Vitamin K hydroquinone formed by the cytosolic, DT-diaphorase (pathway II), would only reach the carboxylase / epoxidase enzyme(s) located in the endoplasmic reticulum of hepatocytes by diffusion, and thus, it has been proposed that under normal circumstances pathway II plays only a minor role in clotting factor synthesis.

Consistent with previous findings in man and experimental animals (Barlow *et al.*, 1982; Hart *et al.*, 1984), the studies described in Chapters 8 and 9 of this thesis illustrated that below a certain dose / plasma concentration, vitamin K_1 from a pharmacological dose cannot be utilized in the vitamin K-epoxide cycle to drive clotting factor synthesis in brodifacoum anticoagulated rabbits and rats. By considering the relationships which exist between the reductase enzymes involved in the vitamin K-epoxide cycle, a possible explanation of this observation can be made. From Figure 10.1 it can be seen that under normal circumstances, vitamin K is conserved and concentrated in the vitamin K-epoxide cycle, vitamin K hydroquinone being produced primarily by pathway I. However, during coumarin

FIGURE 10.1 Proposed relationships between physiological and exogenous vitamin K



$K_{ex.}$ - exogenous vitamin K
$K_{phys.}$ - physiological vitamin K
KH_2 - vitamin K hydroquinone
KO - vitamin K_1 2,3-epoxide
GLU - glutamic acid residues
GLA - γ-carboxygluatmic acid residues
X - inhibition by coumarin anticoagulants

anticoagulation, the enzyme activity(s) vitamin K epoxide reductase and the vitamin K reductase involved in pathway I are inhibited, and as a consequence once the vitamin K associated with the body pool has been depleted, the vitamin K - epoxide cycle has to rely solely on the warfarin insensitive vitamin K reductase (pathway II) for the production of vitamin K hydroquinone for continued clotting factor synthesis. Immediately following a pharmacological dose of vitamin K_1 (10 mg kg^{-1}) to brodifacoum anticoagulated rabbits, plasma concentrations of the vitamin are sufficiently high for it to reach DT-diaphorase in the cytosol of hepatocytes and be converted to vitamin K hydroquinone. Since the vitamin K hydroquinone produced by pathway II, relies on diffusion to reach the carboxylase / epoxidase enzyme(s) in the endoplasmic reticulum, then the more vitamin K hydroquinone produced, the greater the likelihood of some of it reaching its site of action, and thus being utilized in clotting factor synthesis. As plasma concentrations of vitamin K_1 decline rapidly, as a consequence of rapid metabolism in the mitochondria of hepatocytes, then insufficient vitamin K hydroquinone is produced via pathway II to adequately drive clotting factor synthesis.

To investigate further the above proposal, future studies should be directed towards the determination of the relationships between the hepatic sub-cellular concentrations of vitamin K and pharmacological effect, and also the transport mechanisms involved in the movement of vitamin K from plasma to the sub-cellular locations in the liver.

With a view to improving the treatment of coumarin overdosage, it would be of interest to administer the high molecular weight menaquinones, MK7, MK9 and MK10, to coumarin anticoagulated animals to determine their pharmacological activity in this situation. Since

it has been shown that these forms of the vitamin are more active on a molar basis than vitamin K_1 (Wiss and Gloor, 1966; Matschiner and Taggart, 1968), they may be more effective in antagonising the effects of coumarin anticoagulants.

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