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A study of the relationship between the pharmacokinetics and the pharmacodynamics of the 4-hydroxycoumarin anticoagulants warfarin, difenacoum and brodifacoum in the rabbit

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1 The pharmacokinetics and pharmacodynamics of the 4-hydroxycoumarin anticoagulants, brodifacoum, difenacoum, and warfarin have been studied in the rabbit.

2 Sensitive (50 ng ml^{-1}) and specific high performance liquid chromatography assays have been developed for the determination of plasma concentrations of warfarin, brodifacoum and difenacoum.

3 After administration of a single intravenous dose (20 μmol kg^{-1}), plasma concentrations of warfarin underwent mono-exponential decay, with a terminal half-life of 5.6 ± 0.7 h (mean ± s.e. mean), whereas plasma concentrations of brodifacoum and difenacoum underwent bi-exponential decay with terminal half-lives of 60.8 ± 1.9 h and 83.1 ± 10.3 h respectively. The plasma half-life of brodifacoum in a single patient poisoned with the compound was 487 h.

4 The pharmacological response to the anticoagulants was measured as changes in prothrombin complex activity, from which the rate of clotting factor synthesis was determined.

5 Clotting factor synthesis recovered in a monophasic fashion after a single intravenous dose of warfarin, compared with a more complex biphasic, pattern of recovery of clotting factor synthesis after administration of either brodifacoum or difenacoum.

6 The slope (m) of the intensity of effect-log (amount of drug in the body) curve was derived for each anticoagulant. There was no significant difference in the value of m after single intravenous doses of racemic, **R**-, and **S**-warfarin, difenacoum and brodifacoum, which is consistent with the hypothesis that all the 4-hydroxycoumarin anticoagulants produce their anticoagulant effect by acting at the same receptor site, vitamin K epoxide reductase.

7 Determination of the minimum plasma concentration of each anticoagulant that corresponded with the complete inhibition of clotting factor synthesis indicated that racemic warfarin, **R**-warfarin and brodifacoum have similar potencies in the rabbit and are less potent than **S**-warfarin and difenacoum.

Introduction

Difenacoum and brodifacoum are novel 4-hydroxycoumarin anticoagulants which have been developed as rodenticides (Hadler & Shadbolt, 1975). These compounds appear to be more potent anticoagulants than warfarin in man (Barlow *et al.*, 1982), the rat (Hadler & Shadbolt, 1975) and the rabbit (Park & Leck, 1982). In addition difenacoum and brodifacoum are equally effective as rodenticides in warfarin-resistant and warfarin-susceptible rats (Hadler & Shadbolt, 1975).

Warfarin and related 4-hydroxycoumarin anticoagulants are thought to interfere with 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 & Suttie, 1977). The warfarin receptor is thought to be associated with the enzyme vitamin K epoxide reductase. This enzyme is responsible for the continuous regeneration of vitamin K in the physiologically important vitamin K-epoxide cycle (Bell & Matschiner, 1972). Consistent with this hypothesis, it was found that warfarin, difenacoum and brodifacoum produce

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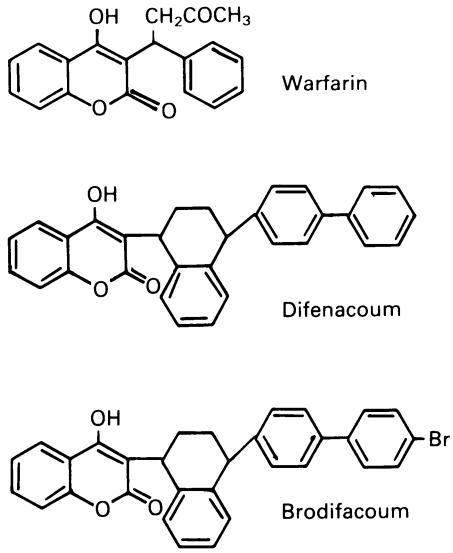

Figure 1 Chemical structures of the 4-hydroxycoumarin anticoagulants.

an accumulation of tritiated vitamin K_1 epoxide in rabbit plasma after intravenous administration of tritiated vitamin K_1 (Park *et al.*, 1979a).

Difenacoum and brodifacoum possess the same 4-hydroxycoumarin ring system as warfarin, but differ chemically in that they both contain a novel tetrahydronaphthyl side chain (Figure 1). It has been suggested that the side chain may provide a point of attachment to a lipophilic site adjacent to the warfarin receptor, to which warfarin does not bind strongly (Hadler & Shadbolt, 1975). However, formal proof of this hypothesis is lacking. We have therfore investigated the pharmacokinetics and pharmacodynamics of warfarin, difenacoum and brodifacoum in order to evaluate the relative contributions of these factors to the pharmacological effect of the anticoagulants observed *in vivo*.

Methods

Male New Zealand White rabbits (2.5–3.0 kg) were used in these studies. The rabbits were maintained on Diet R14, Labsure Animal Foods, Poole; average daily dietary intake of vitamin K_1 was 60 $\mu g\,kg^{-1}$. Racemic, **R**-and **S**-warfarin were obtained from Ward Blenkinsop and were administered intravenously in 0.9% w/v NaCl solution (0.5 $ml\,kg^{-1}$). Difenacoum and brodifacoum were gifts from Sorex Laboratories, Widnes, and were dissolved in polyethylene glycol 200 (B.D.H. Poole, Dorset) for intravenous injection (0.5 $ml\,kg^{-1}$). Vitamin K_1 (Konakion) was a gift from Hoffmann-La Roche, Welwyn Garden City.

Analytical techniques

High performance liquid chromatography The high performance liquid chromatography (h.p.l.c.) system comprised an Altex 110A solvent delivery pump, an Altex 160 fixed wavelength detector, fitted with either a 280 nm or 313 nm filter, and a Philips 8251 single pen chart recorder. All the solvents employed for chromatography were h.p.l.c. grade (Fisons, Loughborough) and were degassed by sonication before use.

Determination of difenacoum and brodifacoum in plasma To determine the concentration of difenacoum and brodifacoum in rabbit and human plasma, a specific and sensitive reversed-phase h.p.l.c. assay was developed. Plasma (1 vol) was extracted twice with methyl-*t*-butyl ether (3 vol) by gentle mechanical tumbling (20 min), after protein precipitation with hydrochloric acid (5 M: 0.5 vol). The organic extracts were pooled, and washed once with an equal volume of sodium hydroxide (0.005 M). After evaporation of the washed extract to dryness under nitrogen at 37 °C, the residue was reconstituted in methyl-*t*-butyl ether (100 μl) and an aliquot (20 μl) chromatographed. The analytical column (25 cm × 4.5 mm i.d.) was packed with Partisil 10 ODS and methanol-water-acetic acid (90:10:1) was used as the mobile phase at a flow rate of 2 ml min^{-1}. Difenacoum and brodifacoum were detected by u.v. absorbance at 280 nm. Difenacoum was employed as the internal standard for the determination of plasma brodifacoum concentrations and brodifacoum was used as the internal standard for the determination of difenacoum in plasma.

The recoveries from plasma at a concentration of 0.5 $\mu g\,ml^{-1}$, were 70–78% and 75–86% for difenacoum and brodifacoum respectively. Retention times were 5.2 min for difenacoum and 7.1 min for brodifacoum, giving a run time of 10 min. Plasma brodifacoum and difenacoum concentrations were determined by the ratio of brodifacoum or difenacoum peak height to that of internal standard. The linear regression line obtained from the standard curve for difenacoum and brodifacoum in rabbit plasma was $y = 1.0454 \times + 0.0165$, $r = 0.998$, at an internal standard concentration of 200 $\mu g\,ml^{-1}$. Intra-assay variation was calculated by the repeated ($n = 10$) analysis of aliquots of a single spiked plasma sample and gave a coefficient of variation of 2.5%. Inter-assay variation was calculated by analysis of aliquots of a single spiked plasma sample on separate days ($n = 10$) over a period of several weeks and gave a coefficient of variation of 9.8%. The limit of sen-

sitivity of the assay for both difenacoum and brodifacoum was 50 ng ml^{-1}.

Determination of warfarin in plasma Plasma warfarin concentrations were determined by normal-phase h.p.l.c. on a Spherisorb 5 μm nitrile column (25 cm × 4.5 mm i.d.) with hexane-isopropanol-dichloromethane - acetic acid (85:10:5:1) as eluent at a flow rate of 2.2 ml min^{-1}. Warfarin was extracted from plasma (1 vol.) with methyl-*t*-butyl ether (2 vol.) by vortexing (3 min), after protein precipitation with hydrochloric acid (5 M; 0.5 vol.). An aliquot (100 μl) of the organic extract was injected onto the analytical column and warfarin was detected by u.v. absorbance at 313 nm. Acenocoumarol was employed as the internal standard.

The extraction efficiency for warfarin from plasma was 95%. Retention times were 5.8 min and 9.4 min for warfarin and acenocoumarol respectively. Plasma warfarin concentrations were determined by the ratio of warfarin peak height to the peak height of internal standard. The linear regression line obtained from the standard curve for warfarin in rabbit plasma was $y = 1.2180 \times + 0.0290$, $r = 0.998$, with an internal standard concentration of 200 μg ml^{-1}. The intra-assay and inter-assay coefficients of variation were 3.1% and 3.9% respectively. The limit of sensitivity of the assay for warfarin in rabbit plasma was 50 ng ml^{-1}.

Determination of cis- *and* trans-*brodifacoum in plasma* Plasma concentrations of *cis*- and *trans*-brodifacoum were determined by the same h.p.l.c. method described for the determination of plasma warfarin. Retention times for *cis*- and *trans*-brodifacoum were 4.2 min and 3.5 min respectively. Although this h.p.l.c. method will separate *cis*-brodifacoum from *trans*-brodifacoum it will not distinguish between *cis*- and *trans*-difenacoum and *cis*- and *trans*-brodifacoum. For each plasma sample the concentration of the individual isomers of brodifacoum was determined from the ratio of the peak heights of *cis*- to *trans*-brodifacoum with reference to the concentration of total brodifacoum in the sample determined by the reversed phase h.p.l.c. method described above.

Determination of prothrombin complex activity Pharmacological response to the 4-hydroxycoumarin anticoagulants was measured by determination of prothrombin complex acitivity (P.C.A.) as previously described (Park *et al.*, 1979a). Blood samples (0.9 ml) were collected into 3.8% w/v trisodium citrate (0.1 ml) in polypropylene tubes and centrifuged (8000 *g* for 2 min) immediately. Thromboplastin (0.1 ml) was added to citrated plasma (0.1 ml) and incubated at 37 °C for 2 min, in duplicate. Calcium chloride (0.025 M; 0.1 ml) was added and the clotting time determined in a Schnitger and Gross coagulometer.

A standard curve of P.C.A. was obtained by determining the clotting times of pooled normal citrated plasma diluted with absorbed plasma (deficient in factors II, VII, IX and X) at concentrations of 1–100%. The P.C.A. for each animal was expressed as a percentage of its own control taking 100% as the beginning of each experiment.

Pharmacokinetic study

Groups of rabbits were dosed (20 μmol kg^{-1}) with either racemic warfarin, difenacoum or brodifacoum via the left marginal ear vein. Blood samples (1–4 ml) were taken from the right marginal ear vein at regular intervals up to 340 h following anticoagulant administration. Plasma was obtained and stored frozen (− 20 °C) until analysed. Animals dosed with either difenacoum or brodifacoum also received 4 mg kg^{-1} vitamin K_1 on alternate days throughout the study to prevent death by haemorrhage. In a pilot study we found that vitamin K_1 administration had no effect upon the pharmacokinetics of difenacoum up to 144 h following anticoagulant administration.

In addition, the plasma concentration-time profile of brodifacoum was studied in a single male patient accidentally poisoned with the compound. The patient received an unknown dose of the compound at an unknown time. The concentration of brodifacoum in five plasma samples taken at regular intervals over a period of twenty two days was determined.

Pharmacodynamic study

Groups of rabbits received either racemic warfarin (2 or 20 μmol kg^{-1}), **R**-warfarin (2 μmol kg^{-1}), **S**-warfarin (2 μmol kg^{-1}), difenacoum (0.5 or 1 μmol kg^{-1}) or brodifacoum (0.1 μmol kg^{-1}) intravenously into the left marginal ear vein. Serial blood samples were taken from the right marginal ear vein for the immediate determination of prothrombin time (1 ml) and subsequent determination of plasma anticoagulant concentrations (1–4 ml).

Efect of phenobarbitone on the pharmacological response to difenacoum in rabbits

The effect of phenobarbitone pretreatment on the pharmacological response to difenacoum in rabbits was investigated. Rabbits received either phenobarbitone (40 mg kg^{-1} daily for 4 days) or saline (2 ml kg^{-1} daily for 4 days) intraperitoneally. This phenobarbitone dosing schedule had previously been shown to induce the hepatic mixed function oxidase system in the rabbit (Wilson & Park, 1984). On the

fifth day of the study all rabbits received a single dose of difenacoum (1 μmol kg^{-1}) into the left marginal ear vein. Blood samples (1 ml) were taken at regular intervals up to 384 h following difenacoum administration for the immediate determination of P.C.A.

Pharmacokinetic analysis

The plasma concentration-time data following single intravenous doses of brodifacoum and difenacoum, were fitted to a bi-exponential equation using a regression analysis programme (Nielsen-Kudsk, 1980). In each case the correlation coefficient for the two first order rate constants was greater than 0.96. 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 exponent. Pharmacokinetic parameters were defined for brodifacoum and difenacoum assuming a two-compartment model and for warfarin assuming a one-compartment model: AUC = area under the plasma concentration-time curve to infinity; β = elimination rate constant; apparent volume of distribution (V_d) = dose/(A.U.C. $\times$ β); plasma clearance (Cl_p) = $V_d \times \beta$.

Statistical analysis of results

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

Results

Analysis of the 4-hydroxycoumarins in plasma

Sensitive and specific h.p.l.c. assays have been developed for the determination of warfarin, difenacoum and brodifacoum in rabbit and human plasma. Satisfactory separation from endogenous plasma components was achieved with normal-phase chromatography for warfarin (Figure 2A), and by

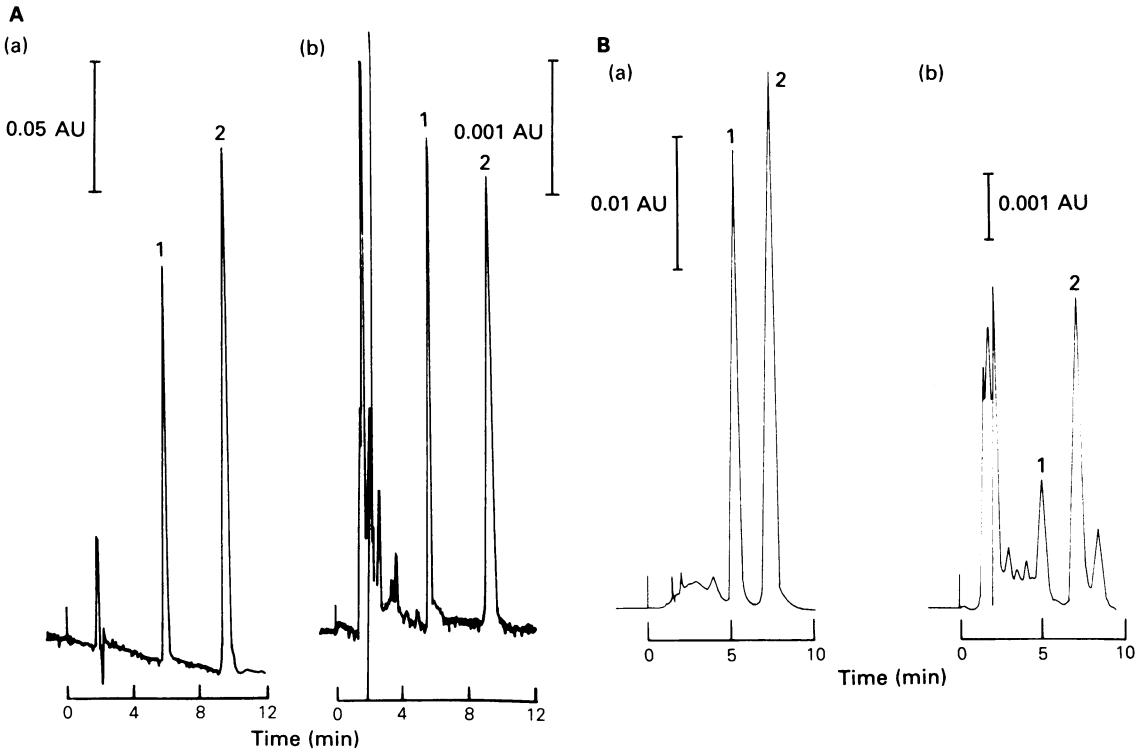

Figure 2 (A) Determination of warfarin in rabbit plasma: (a) separation of 250 ng warfarin and acenocoumarol, (b) extract from 1 ml plasma containing 250 ng warfarin. Peaks: 1 = warfarin; 2 = acenocoumarol. (B) Determination of brodifacoum and difenacoum in rabbit plasma: (a) separation of 500 ng difenacoum and brodifacoum, (b) extract from 1 ml plasma 240 h following intravenous administration of brodifacoum (20 μmol kg^{-1}). Peaks: 1 = difenacoum (internal standard); 2 = brodifacoum.

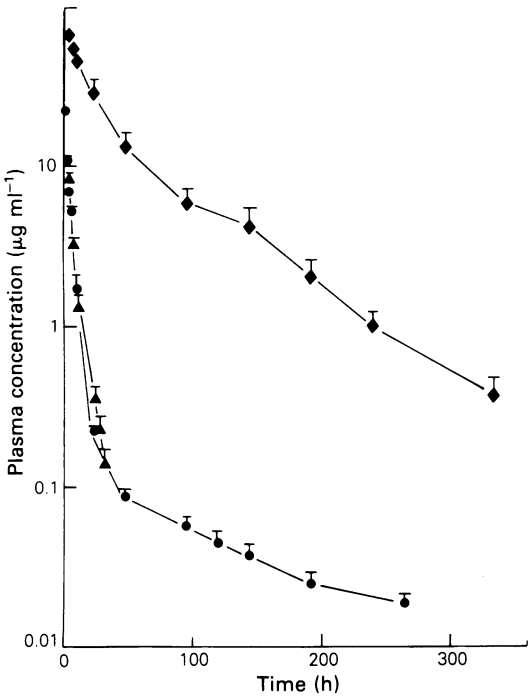

Figure 3 Plasma concentrations of 4-hydroxycoumarins vs. time in rabbits after intravenous administration (20 μmol kg^{-1}) of warfarin (▲; $n=8$), difenacoum (●; $n=4$) and brodifacoum (◆; $n=4$). Results are presented as means with vertical lines showing s.e. mean.

reversed-phase chromatography for difenacoum and brodifacoum (Figure 2B).

Pharmacokinetics of the 4-hydroxycoumarins in the rabbit

The plasma concentration-time curves following intravenous administration of single equimolar doses (20 μmol kg^{-1}) of racemic warfarin, difenacoum and brodifacoum in the rabbit are shown in Figure 3. The plasma concentration of racemic warfarin fell monoexponentially with time, and thus the drug was distributed in a single compartment. During the first 36 h following administration of difenacoum the plasma

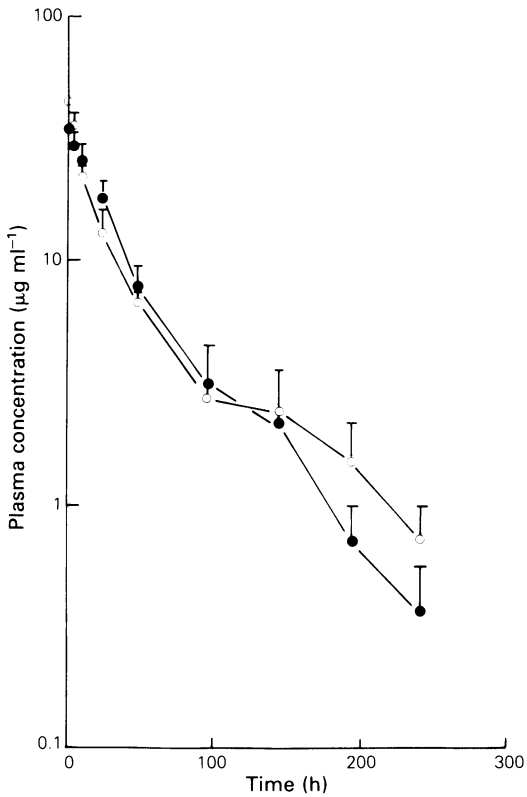

Figure 4 Plasma concentrations of *cis*-brodifacoum (●) and *trans*-brodifacoum (○) in rabbits after intravenous administration of a single dose (20 μmol kg^{-1}) of the isomer mixture. Results are presented as means ($n=4$) and vertical lines show s.e. mean.

Table 1 Pharmacokinetic parameters of warfarin, difenacoum and brodifacoum in the rabbit

	Dose (μmol kg^{-1})	$t_{1}\alpha$ (h)	$t_{1}\beta$ (h)	Cl_p (ml min^{-1} kg^{-1})	V_d (1 kg^{-1})
Racemic warfarin ($n=8$)	20	–	5.60 ± 0.65	1.33 ± 0.08	0.65 ± 0.10
Racemic warfarin ($n=4$)	2	–	7.65 ± 2.19	1.17 ± 0.12	0.70 ± 0.12
Difenacoum ($n=4$)	20	3.20 ± 0.08	83.10 ± 10.31	1.43 ± 0.09	10.35 ± 1.44
Brodifacoum ($n=4$)	20	12.68 ± 0.58	60.84 ± 1.87	0.08 ± 0.02	0.41 ± 0.10
R-Warfarin ($n=4$)	2	–	6.39 ± 0.18	0.73 ± 0.04	0.41 ± 0.03
S-Warfarin ($n=4$)	2	–	4.27 ± 0.28	2.82 ± 0.27	0.96 ± 0.09

Values show mean ± s.e. mean. $t_{\frac{1}{2}}\alpha$ = plasma half-life; $t_{\frac{1}{2}}\beta$ = terminal plasma half-life; Cl_p = plasma clearance; V_d = apparent volume of distribution.

concentration-time curve was similar to that of warfarin. However, a second elimination phase with a much longer half-life was detected (Table 1). The plasma concentration of brodifacoum also declined in a bi-exponential manner with a long terminal half-life (Table 1); brodifacoum concentrations were always significantly ($P<0.01$) greater than those of warfarin and difenacoum. The pharmacokinetic parameters calculated from these data are shown in Table 1. In the rabbit, the terminal half-lives of difenacoum (83.1 ± 10.3 h) and brodifacoum (60.8 ± 1.9 h) were approximately ten fold greater than for racemic warfarin (5.6 ± 0.8 h). Brodifacoum was cleared from plasma more slowly (0.08 ± 0.02 ml min^{-1} kg^{-1}) than warfarin (1.33 ± 0.08 ml min^{-1} kg^{-1}), whereas difenacoum had a far greater apparent volume of distribution (10.4 ± 1.4 l kg^{-1}) compared with warfarin (0.65 ± 0.10 l kg^{-1}). The plasma half-life of brodifacoum in man determined in a single patient poisoned with the compound was 487 h. This plasma half-life was again approximately ten fold greater than that found for racemic warfarin in man (O'Reilly *et al.*, 1963; O'Reilly & Aggeler, 1968).

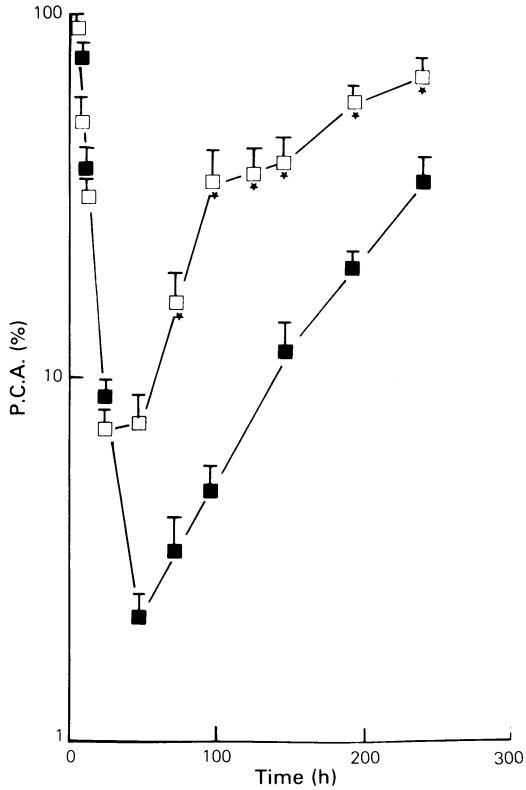

Figure 6 Prothrombin complex activity (P.C.A.) vs. time following intravenous administration of difenacoum (1 μmol kg^{-1}); difenacoum in control (■) and phenobarbitone-pretreated (□) rabbits. Results are presented as means ($n=4$) and vertical lines show s.e. mean; $^*P<0.05$.

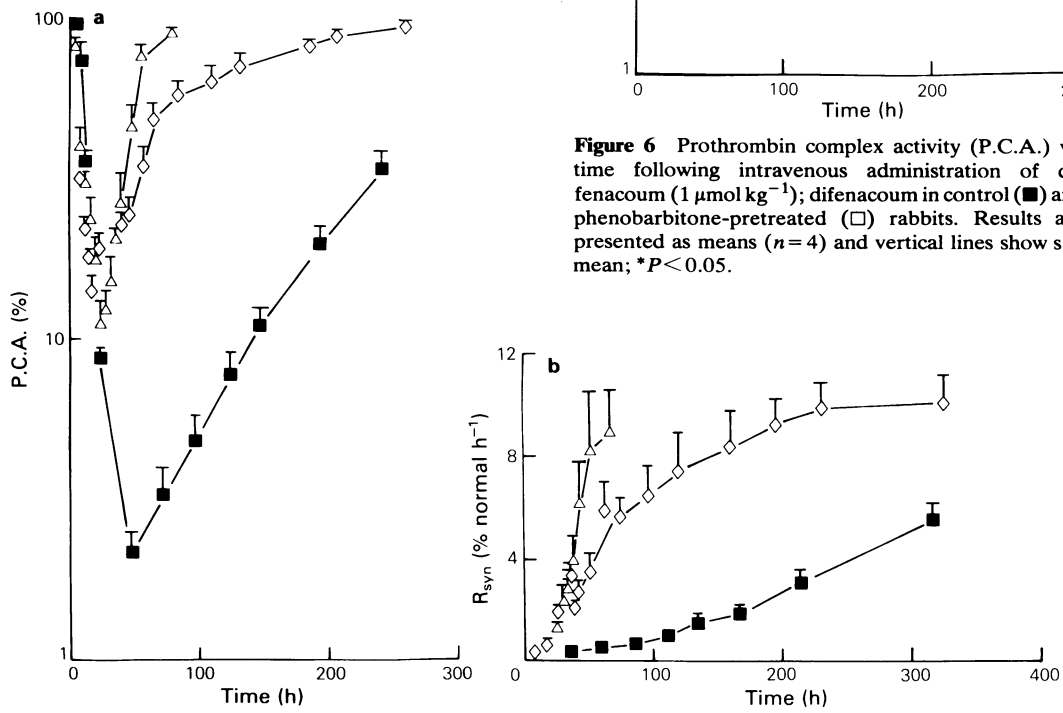

Figure 5 (a) Prothrombin complex activity (P.C.A.) vs. time in rabbits following single intravenous doses of 2 μmol kg^{-1} racemic warfarin (△), 0.1 μmol kg^{-1} brodifacoum (◇) and 1 μmol kg^{-1} difenacoum (■). (b) Rate of prothrombin complex activity synthesis vs. time in rabbits following single intravenous doses of 2 μmol kg^{-1} warfarin (△), 0.1 μmol kg^{-1} brodifacoum (◇) and 1 μmol kg^{-1} difenacoum (■). Results are presented as means ($n=4$) and vertical lines show s.e. mean.

Commercially available difenacoum and brodifacoum are a mixture of *cis*- and *trans*-isomers which we were able to separate by normal-phase chromatography. The plasma concentration-time profiles of *cis*- and *trans*- brodifacoum, determined in the same rabbits which received 20 μmol kg^{-1} of the mixture of brodifacoum isomers, are shown in Figure 4. There was no significant difference between *cis*- and *trans*-brodifacoum concentrations up to 240 h following administration of the isomer mixture.

The plasma concentration-time curves following a single intravenous dose of racemic warfarin, **R**-warfarin and **S**-warfarin (2 μmol kg^{-1}) were studied in separate groups of rabbits. Following a lower dose (2 μmol kg^{-1}) of racemic warfarin the pharmacokinetic parameters were unchanged (Table 1), indicating that the pharmacokinetics of racemic warfarin were independent of dose over a tenfold dose range. The pharmacokinetics of difenacoum and brodifacoum could only be studied at the relatively high dose of 20 μmol kg^{-1} because these compounds have very long elimination half-lives (Table 1). The plasma half-life of **S**-warfarin in rabbits was found to be significantly ($P<0.001$) shorter than that of **R**-warfarin. **S**-warfarin was cleared from plasma approximately 4 times more rapidly than **R**-warfarin (Table 1).

Pharmacodynamics of the 4-hydroxycoumarins in the rabbit

In order to investigate the duration of action of the 4-hydroxycoumarin anticoagulants it was necessary to determine, for each individual anticoagulant, a dose which would initially produce maximum inhibition of clotting factor synthesis for at least 18 h, but from which recovery could be monitored without the administration of vitamin K_1. Thus the duration of action of the 4-hydroxycoumarins had to be studied at different doses for each anticoagulant (Figure 5).

The initial half-lives of degradation of P.C.A. after administration of 2 μmol kg^{-1} and 20 μmol kg^{-1} racemic warfarin (6.8 ± 0.3 h and 6.6 ± 0.5 h respectively), 0.1 μmol kg^{-1} brodifacoum (6.8 ± 0.6 h), and 0.5 μmol kg^{-1} and 1 μmol kg^{-1} difenacoum (6.7 ± 0.2 h and 7.4 ± 0.8 h, respectively) all corresponded to the maximum rate of decay of P.C.A. and therefore indicated complete inhibition of clotting factor synthesis (Park *et al.*, 1979a, b).

The effect of phenobarbitone pretreatment on the duration of action of difenacoum, following a single intravenous dose (1 μmol kg^{-1}), is shown in Figure 6. Initially P.C.A. declined at a maximum rate in both the phenobarbitone-pretreated and control rabbits. The half-life of degradation of P.C.A. was 6.1 ± 0.5 h and 7.4 ± 0.8 h respectively. However P.C.A. began to return to normal 24 h after difenacoum administration in phenobarbitone-pretreated animals compared with 48 h in control animals. P.C.A. remained significantly ($P<0.05$) higher in the phenobarbitone pretreated rabbits compared with controls at all times from 72 h following difenacoum administration (Figure 6). In addition 2 out of 6 of the control rabbits died from haemorrhage during the study whereas there were no deaths in the phenobarbitone-pretreated group.

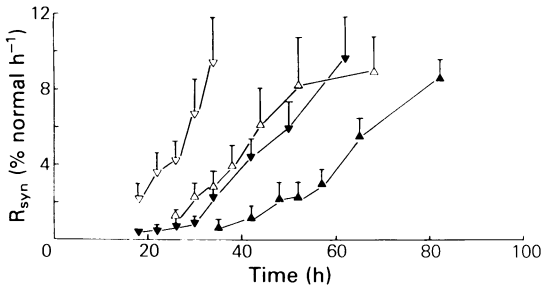

Figure 7 Rate of prothrombin complex activity synthesis vs. time in rabbits follwoing intravenous administration of 20 μmol kg^{-1} racemic warfarin (▲), 2 μmol kg^{-1} racemic warfarin (△), 2 μmol kg^{-1} **R**-warfarin (▼) and 2 μmol kg^{-1} **S**-warfarin (▽). Results are presented as means ($n=4$) and vertical lines show s.e. mean.

There is no direct relationship between P.C.A. and the plasma concentration of 4-hydroxycoumarin anticoagulants 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)

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

Coumarin anticoagulants only affect clotting factor synthesis (Aggeler & O'Reilly, 1966). Thus R_{syn} is a better pharmacological end point than P.C.A. for 4-hydroxycoumarin anticoagulants. The rate constant of degradation of P.C.A. (K_d) may be determined for each animal when degradation of P.C.A. is maximal i.e. during the first 18 h following warfarin, difenacoum or brodifacoum administration (Figure 5a). Therefore, the rate of P.C.A. synthesis may be calculated at various times following anticoagulant administration according to equation 2 (Nagashima *et al.*, 1969)

$$R_{syn} = R_{net} + K_d.P \qquad (2)$$

The duration of action of warfarin, difenacoum and brodifacoum is illustrated in Figures 5b, 7 and 8, in which R_{syn} is plotted against time. According to current concepts, the anticoagulant effect of the 4-hydroxycoumarins will subside when the concentration of the drug at the receptor associated with vitamin K expoxide reductase falls (Bell, 1978; Park *et al.*, 1979a). Theoretically, for a drug distributed in a single compartment, and eliminated by first order

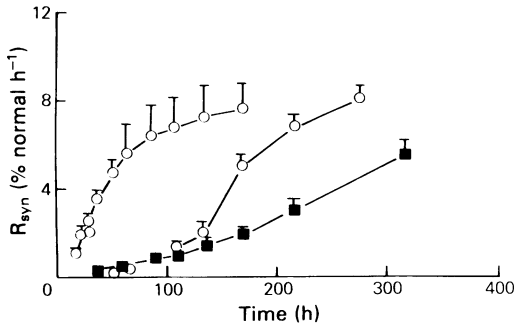

Figure 8 Rate of prothrombin complex activity synthesis vs. time in rabbits following intravenous administration of 1 μmol kg^{-1} difenacoum (■; $n=4$) and 0.5 μmol kg^{-1} difenacoum (○; $n=3$ for each group). Results are presented as means and vertical lines show s.e. mean.

kinetics the intensity of effect (I) is given by the following equation (Rowland & Tozer, 1980):

$$I = I_o - \frac{m.k.t}{2.3} \qquad (3)$$

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 and the elimination half-life of the drug according to the equation:

$$\Delta I = \frac{m.k}{2.3} \qquad (4)$$

The advantage of this type of analysis is that a distinction between receptor interaction (pharmacodynamics) and rate of elimination (pharmacokinetics) as a cause for prolongation of effect can be made. It was therefore of interest to apply this approach to the 4-hydroxycoumarin anticoagulants.

After intravenous administration of warfarin, plasma concentrations fell mono-exponentially (Figure 3, Table 1); the drug was distributed in a single compartment; and the pharmacological effect (depression of R_{syn}; Figure 7) fell linearly with time. Therefore, by substitution of the plasma elimination rate constant (k), and the rate of decline of intensity of effect (Δ I) into equation 4, the slope (m) of the intensity-log (amount of drug in the body) curve may be calculated. For racemic warfarin the value for m was the same after administration of 2 μmol kg^{-1} (48.3 ± 11.6) and after 20 μmol kg^{-1} (50.3 ± 9.9); furthermore similar values were obtained for **R**-warfarin (63.3 ± 14.0) and **S**-warfarin (46.5 ± 8.2).

After intravenous administration of difenacoum (1 μmol kg^{-1}), R_{syn} recovered monophasically with time (Figure 8). We were unable to determine the plasma pharmacokinetics of difenacoum after such a low dose. However, if we use the mean terminal elimination rate constant obtained at a higher dose (20 μmol kg^{-1}) of difenacoum, a value for m is obtained (60.1 ± 2.5) which is not significantly different from that obtained for warfarin. After administration of a lower dose of difenacoum (0.5 μmol kg^{-1}), a more complicated pattern of recovery of R_{syn} was observed (Figure 8). First, there was a very wide inter-animal variation in the onset of recovery and the animals appeared to fall into two distinct groups. Secondly, R_{syn} underwent biphasic recovery.

After intravenous administration of brodifacoum (0.1 μmol kg^{-1}) R_{syn} underwent a biphasic recovery (Figure 5b). The initial phase of recovery was similar to that observed with warfarin, in contrast to the second phase of the recovery of R_{syn} which was significantly ($P<0.01$) slower. Thus it appears that brodifacoum undergoes bi-exponential elimination from both plasma and the receptor. Calculation of m from Δ I and the mean α plasma elimination rate constant obtained earlier (Table 1) gave a value 45.5 ± 8.4, which was not significantly different from that obtained with either warfarin or difenacoum. After administration of a higher dose of brodifacoum

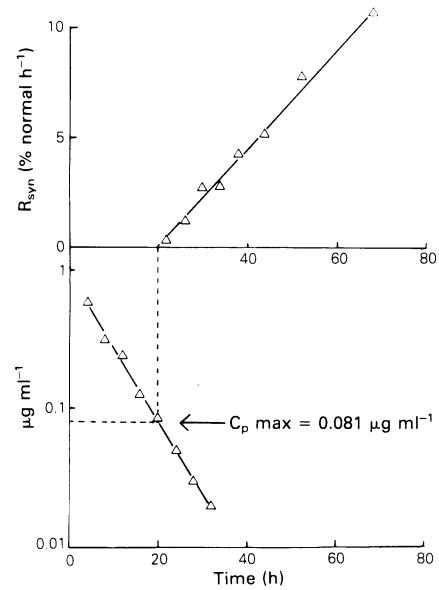

Figure 9 Graphical determination of the minimum plasma concentration of anticoagulant that corresponds with the complete inhibition of clothing factor synthesis ($C_p$max). Data for an individual rabbit following 2 μmol kg^{-1} racemic warfarin.

($0.2\,\mu mol\,kg^{-1}$) P.C.A. declined to $<5\%$ control values, and vitamin K administration was necessary to return P.C.A. to normal.

Relationship between the pharmacokinetics and pharmacodynamics of the 4-hydroxycoumarins

The relationship between the pharmacokinetics (plasma concentration-time curve) and the pharmacodynamics (R_{syn}-time curve) was determined following each of the 4-hydroxycoumarin anticoagulants, for individual animals, by the graphical method illustrated in Figure 9. Combined data cannot be used for such an analysis (Nagashima *et al.*, 1969). Using this procedure we determined the minimum plasma concentration of each of the anticoagulants that corresponded with the complete inhibition of clotting factor synthesis ($C_p max$) in the rabbit. On the basis of these determinations it would appear that brodifacoum ($C_p max = 0.067 \pm 0.005\,\mu g\,ml^{-1}$), racemic warfarin ($C_p max = 0.103 \pm 0.031\,\mu g\,ml^{-1}$) and **R**-warfarin ($C_p max = 0.099 \pm 0.018\,\mu g\,ml^{-1}$) have similar potencies and are less potent than **S**-warfarin ($C_p max \leqslant 0.025\,\mu g\,ml^{-1}$) and difenacoum ($C_p max \leqslant 0.025\,\mu g\,ml^{-1}$). The $C_p max$ for **S**-warfarin and difenacoum were below the limit of sensitivity of our analytical technique.

Discussion

Brodifacoum and difenacoum have the same mechanism of action as warfarin and block clotting factor synthesis indirectly, by interruption of the vitamin K-epoxide cycle at the epoxide reductase enzyme (Park *et al.*, 1979a; Leck & Park, 1981). However, difenacoum and brodifacoum are far more powerful anticoagulants than warfarin in a number of species, including man, the rat and the rabbit (Barlow *et al.*, 1982; Hadler & Shadbolt, 1975; Park & Leck, 1982). It has been suggested that the large lipophilic side chain present in brodifacoum and difenacoum may enhance their affinity for the warfarin receptor (Hadler & Shadbolt, 1975), but formal proof of this hypothesis is lacking. The purpose of this study was to design an experimental model with which to investigate and compare the pharmacokinetic and pharmacodynamic characteristics of 4-hydroxycoumarin anticoagulants. The rabbit was selected as an animal model because the dynamics and kinetics of anticoagulants may be determined simultaneously in the same animal over a period of several days.

Pharmacokinetics of the 4-hydroxycoumarin anticoagulants

After intravenous administration of a single equimolar dose, plasma concentrations of warfarin declined mono-exponentially, whereas plasma concentrations of brodifacoum and difenacoum exhibited a bi-exponential decay. The plasma half-life for racemic warfarin in the rabbit (6.6 h) is similar to that in the rat (5.3 h; Yacobi *et al.*, 1974) but shorter than that reported for man (15–80 h; O'Reilly *et al.*, 1963; O'Reilly & Aggeler, 1968). The terminal half-lives ($t_{\frac{1}{2}\beta}$) for brodifacoum and difenacoum in the rabbit were approximately tenfold greater than that of warfarin (Table 1, Figure 3). The serum half-life of brodifacoum in the rat has been calculated to be 156 h (Bachmann & Sullivan, 1983). Furthermore; the plasma half-life for brodifacoum of 487 h, determined in a single human subject, is also approximately ten times greater than the average plasma warfarin half-life in man. Thus the tetrahydronaphthyl side chain of brodifacoum and difenacoum appears to markedly increase the plasma half-lives of these compounds, compared with warfarin, in man and the rabbit.

Half-life is a hybrid term and is dependent upon plasma clearance (Cl_p) and apparent volume of distribution (V_d). Further pharmacokinetic analysis revealed that difenacoum and brodifacoum have longer terminal half-lives than warfarin, but for different reasons. Difenacoum has a far greater apparent volume of distribution than warfarin but a similar plasma clearance; brodifacoum, in contrast, has a similar apparent volume of distribution to warfarin but a greatly reduced plasma clearance. It is interesting to note that simple bromo-substitution should have such a pronounced effect upon the disposition of the tetrahydronaphtyl anticoagulants.

Stereochemical factors are also important determinants of the disposition of 4-hydroxycoumarin anticoagulants (Breckenridge & Orme, 1972; Breckenridge *et al.*, 1974). In the rabbit **S**-warfarin was cleared more rapidly than **R**-warfarin (Table 1), in keeping with results obtained in man (Wingard *et al.*, 1978). In addition, we found that the pharmacokinetics of racemic warfarin in the rabbit were independent of dose over the dose range investigated. Commercial brodifacoum and difenacoum are available as a mixutre of *cis*- and *trans*-isomers (Koubek *et al*, 1979). It was therefore of interest to determine the importance of the stereochemistry of the tetrahydronaphthyl group with regard to the disposition of these compounds. Pharmacokinetic analysis indicated that the isomers had similar distribution and elimination rates (Figure 4).

Pharmacodynamics of the 4-hydroxycoumarin anticoagulants

There is no direct relationship between prothrombin complex activity (P.C.A.) and the plasma concentra-

tion of coumarin anticoagulants such as warfarin (Nagashima *et al.*, 1969). Therefore, the determination of the rate of synthesis of P.C.A. (R_{syn}), rather than the net rate of change in P.C.A., is a better pharmacological end point with which to investigate the pharmacological response to 4-hydroxycoumarin anticoagulants.

Complete inhibition of clotting factor synthesis, followed by a natural recovery of P.C.A. (without recourse to vitamin K_1 administration), after a single intravenous dose was achieved over a ten fold dose range for warfarin ($2-20\ \mu mol\ kg^{-1}$). This could only be achieved over a two fold dose range with difenacoum ($0.5-1\ \mu mol\ kg^{-1}$) and for only a single dose of brodifacoum ($0.1\ \mu mol\ kg^{-1}$). A two fold increase in the dose of brodifacoum ($0.2\ \mu mol\ kg^{-1}$) produced death from haemorrhage. These findings alone illustrate the greater potential toxicity of difenacoum and brodifacoum compared with warfarin.

Synthesis of P.C.A. (R_{syn}) recovered monophasically after administration of racemic, **R**- and **S**-warfarin, at a rate which was independent of dose (Figure 7). The recovery of R_{syn} after anticoagulation with brodifacoum and difenacoum was more complex (Figure 5b). This may be a reflection of the more complex pharmacokinetic profiles of difenacoum and brodifacoum compared with warfarin. In each case there was evidence for a biphasic recovery of R_{syn}. In addition, the pattern of recovery of R_{syn} was also dose-dependent for difenacoum (Figure 8). Therefore, after poisoning with these novel 4-hydroxycoumarin anticoagulants, the rate and time course of recovery of clotting factor synthesis is difficult to predict.

The recovery of clotting factor synthesis after a single intravenous dose of difenacoum was more rapid in rabbits induced with phenobarbitone. Phenobarbitone pretreatment has been shown to increase the rate of metabolism of warfarin and thereby decrease its pharmacological effect in a number of species, including man (Breckenridge *et al.*, 1973). Therefore, it is probable that metabolism is the rate-limiting step in the clearance of difenacoum from the receptor. Indeed, enzyme induction may be useful in the treatment of poisoning with this kind of anticoagulant. In keeping with this hypothesis we found that both pre- and post-treatment with phenobarbitone increased the LD_{50} of difenacoum two fold in the mouse (Park & Hart, unpublished data).

Relationship between the pharmacokinetics and pharmacodynamics of the 4-hydroxycoumrins

It would appear that the biphasic elimination of difenacoum and brodifacoum from plasma is mirrored by a similar biphasic elimination of the anticoagulants from the receptor, vitamin K epoxide reductase. 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 distinguish the contribution of these two processes in determining the prolonged action of difenacoum and brodifacoum, we have calculated m, the slope of the intensity-log (amount of drug in the body) curve for each of the anticoagulants. For this purpose we have determined m from Δ I, the rate of decline of intensity of anticoagulant effect, and from k, the rate of elimination of the anticoagulants from plasma. There was no significant difference in m for racemic, **R** and **S**-warfarin, difenacoum and brodifacoum. This similarity in m values indicates that the 4-hydroxycoumarins act at the same receptor and that the slow rate of recovery of R_{syn}, after either difenacoum or brodifacoum administration, is simply a consequence of their pharmacokinetic properties.

Determination of the miniumum plasma concentration of each anticoagulant that completely inhibits clotting factor synthesis ($C_p max$) in the rabbit suggested that racemic warfarin **R**-warfarin and brodifacoum have similar potencies and are less potent than **S**-warfarin and difenacoum. The approach we have used in determining the relative potencies of the 4-hydroxycoumarin anticoagulants makes the fundamental assumption that there is simple equilibration of these compounds between plasma and receptor. However Takada & Levy (1979) found that the hepatic uptake of warfarin is concentration-dependent in the rat. Subsequently it has been shown that there are at least two classes of hepatic tissue exchanging warfarin with plasma, and that one of these tissues exhibits Michaelis-Menten saturation kinetics (Covell *et al.*, 1983). Thus determination of the minimum liver concentrations of the 4-hydroxycoumarins required to inhibit completely clotting factor synthesis is necessary for a more accurate indication of the relative pharmacological potencies of these compounds. Nevertheless, it is important, from a toxicological point of view, to establish the range of plasma concentrations over which the 4-hydroxycoumarins may be active.

In conclusion, we have developed an experimental model with which to investigate and compare the pharmacokinetics and pharmacodynamics of the 4-hydroxycoumarin anticoagulants, in order to evaluate the relative contributions of these factors to the pharmacological effect of the compounds observed *in vivo*. Our findings suggest that it is primarily differences between the pharmacokinetics of difenacoum and brodifacoum, compared with warfarin, which explain the greater duration of action of these tetrahydronaphthyl anticoagulants. It is also important to note that the rate of recovery of clotting factor synthesis, after administration of either di-

fenacoum or brodifacoum, is dependent on dose and may be biphasic. Thus in cases of poisoning with these compounds, P.C.A. should be monitored for a much greater period of time than would normally be required after warfarin poisoning (Barlow *et al.*, 1982).

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