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A STUDY OF FACTORS WHICH DETERMINE THE PHARMACOLOGICAL RESPONSE TO VITAMIN K IN COUMARIN ANTICOAGULATED RABBITS

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Abstract—The pharmacological response to vitamin K has been determined by measuring prothrombin complex acitivity (P.C.A.) in male New Zealand White rabbits anticoagulated (P.C.A. < 20%) with the long acting 4-hydroxycoumarin brodifacoum, at a dose (10 mg/kg) which produces maximum antagonism of vitamin K_1. Thus, according to current concepts, this animal model may be used to assess vitamin K requirements in the absence of a functional vitamin K–epoxide reductase. After intravenous administration of vitamin K_1 (1 mg/kg) P.C.A. reached a maximum (64 ± 19%) at 3 hr and then declined at a rate which corresponds to complete inhibition of clotting factor synthesis. Vitamin K_2 (1 mg/kg) stimulated clotting factor synthesis for 2 hr, while *cis*-vitamin K_1, vitamin K_3, vitamin K_1 2,3-epoxide and oral administration of vitamin K_1 were ineffective. Plasma concentrations of vitamin K_1 fell steeply during the 12 hr following administration of a pharmacological dose, and then declined with a terminal half-life of 18.9 ± 9.0 hr. Comparison of the pharmacodynamic and pharmacokinetic data indicated that plasma concentrations in the range 0.4–1.0 μg/ml are required for clotting factor synthesis in the limiting situation of maximum antagonism of vitamin K by coumarin anticoagulants. These findings explain why frequent and repeated administration of vitamin K_1 may be necessary during coumarin poisoning.

Vitamin K is required for the post-translational modification of glutamyl residues to γ-carboxyglutamyl residues in precursor proteins for clotting factors II, VII, IX, and X [1]. It is thought that vitamin K is reduced to vitamin K hydroquinone which is then converted to vitamin K 2,3-epoxide during the γ-carboxylation process. The carboxylation of glutamyl residues appears to be coupled to the epoxidation of the vitamin [2], although the two processes may be dissociated under certain conditions [3]. The 2,3-epoxide is reduced back to vitamin K by a microsomal epoxide reductase during normal clotting factor synthesis [4–6]. It has been suggested that a single enzyme may catalyze the reduction of both vitamin K quinone and vitamin K 2,3-epoxide [7]. The cyclic interconversion of vitamin K and vitamin K 2,3-epoxide is referred to as the vitamin K–epoxide cycle. The pool of vitamin K associated with clotting factor synthesis is thought to be small [8].

Interruption of the vitamin K–epoxide cycle, at either the epoxidase/carboxylase or the epoxide reductase, inhibits clotting factor synthesis [9]. Thus coumarin anticoagulants inhibit the epoxide reductase whereas direct antagonists of vitamin K, such as 2-chlorovitamin K, inhibit the epoxidase/carboxylase system.

Normal daily requirements for the vitamin are low (*ca.* 1 μg/kg) [10]. However, vitamin K_1 administration, in pharmacological doses, is required after intentional or accidental poisoning with coumarin anticoagulants. In such circumstances, the duration of action of the vitamin may be short and therefore repeated and frequent administration of the vitamin is necessary [11–13]. There is no information available which relates dose and route of administration to pharmacological effect. Furthermore, plasma concentration–pharmacological effect relationships for the vitamin have not been determined. Therefore, we have investigated the disposition of vitamin K in relation to its pharmacological response in coumarin anticoagulated rabbits, with particular reference to the dose, route of administration and chemical structure of the vitamin. In this respect, the coumarin anticoagulant brodifacoum provides a useful pharmacological tool for the maintenance of maximum vitamin K antagonism [14].

MATERIALS AND METHODS

Male New Zealand White rabbits (2.5–3.0 kg) were used in these studies. The rabbits had free access to food and water throughout the experiments and were maintained on Diet R14 (Labshore Animal Foods, Poole, U.K.). The average daily intake of vitamin K_1 was *ca.* 60 μg/kg.

Vitamin K_1 (Konakion®) was obtained from Hoffmann La Roche, Welwyn Garden City. Vitamin K_2, vitamin K_3, and *cis*-vitamin K were all gifts from Hoffmann La Roche. Vitamin K 2,3-epoxide was synthesized according to the method of Tischler *et al.* [15]. Brodifacoum (3-[3(4^1-bromo(1,1^1-biphenyl)-4-yl-1,2,3,4-tetrahydro-1-naphthalenyl]-4-hydroxy-2H-1-benzopyran-2-one) and 2-chloro-3-phytyl-1,4-naphthoquinone (2-chlorovitamin K) were gifts from Sorex Laboratories, Widnes. For intravenous injections brodifacoum was dissolved in polyethylene glycol 200 (0.5 ml/kg) and warfarin in saline (0.5 ml/

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kg). The vitamin K analogues and 2-chlorovitamin K were dissolved in Tween 80 and diluted with 0.9% saline to solutions containing 5% Tween. For intravenous administration of the vitamin K analogues 0.2 ml/kg of the final solution was given. 2-Chlorovitamin K was also administered intravenously and intraperitoneally (10 ml/kg) in 5% Tween-saline. For oral administration of vitamin K_1, Konakion® was diluted to 2 ml/kg with water. Thromboplastin was obtained from the National (U.K.) Reference Laboratory for Anticoagulant Reagents and Control, Manchester. All solvents used were h.p.l.c. grade (Fisons, Loughborough) and all other reagents were of analytical grade (B.D.H. Poole, Dorset).

General plan of study. Before the start of each experiment the control prothrombin complex activity (P.C.A.) for each rabbit was determined. Rabbits were then dosed with either brodifacoum (10 mg/kg, i.v. at t = −24 hr); warfarin (63 mg/kg i.p. at t = −32 hr, −24 hr, −16 hr, −8 hr and at 0 hr), or 2-chlorovitamin K (10 mg/kg i.p. at t = −18 hr, t = −12 hr and i.v. at t = −0.5 hr). P.C.A. was then determined again for each rabbit immediately prior to vitamin K administration to ensure P.C.A. was less than 20% of control values. At t = 0 hr vitamin K_1 (or a structural analogue) was administered and blood samples were taken at regular intervals for the immediate determination of P.C.A. (0.9 ml) and subsequent determination of plasma *trans*-vitamin K_1 and vitamin K_1 2,3-epoxide (4 ml). All intravenous injections were via the left marginal ear vein and all blood samples were removed from the right marginal ear vein.

Using this experimental system, we investigated the pharmacological response in brodifacoum (10 mg/kg) anticoagulated rabbits after intravenous administration of vitamin K_1 (1 and 10 mg/kg) vitamin K_2 (1 mg/kg), vitamin K_3 (2.5 mg/kg), *cis*-vitamin K_1 (1 mg/kg), vitamin K_1 2,3-epoxide (1 mg/kg) and after oral administration of vitamin K_1 (1 mg and 10 mg). In experiments where vitamin K_1 was given, *trans*-vitamin K_1 was measured at regular intervals in order to determine plasma concentration–pharmacological response relationships for the vitamin. The pharmacological response to vitamin K_1 (10 mg/kg) in animals anticoagulated with 2-chlorovitamin K was investigated using the same experimental system.

In addition the plasma concentration–time profile of *trans*-vitamin K_1 was studied in detail up to 32 hr after intravenous administration of Konakion® (10 mg/kg) in control rabbits. Pharmacokinetic analysis was performed using a weighted nonlinear least squares regression method (NONLIN).

Prothrombin complex activity. Prothombin complex activity (P.C.A.) was determined as previously described [16]. Blood samples (0.9 ml) were collected into 3.8% trisodium citrate (0.1 ml) in polypropylene tubes and immediately centrifuged (8000 *g* for 2 min). Thromboplastin (0.1 ml) was added to citrated plasma (0.1 ml) and incubated at 37° for 2 min, in duplicate. 0.025 M calcium chloride (0.1 ml) was added and the clotting times determined in a Schnitger and Gross coagulometer. A standard curve of P.C.A. was obtained by determining the prothrombin times of pooled normal citrated plasma diluted with absorbed plasma (deficient in factors II, VII, IX and X) at concentrations of 1–100 per cent. The P.C.A. for each animal was expressed as a percentage of its own control taking 100% as the beginning of each experiment.

Plasma concentrations of trans-*vitamin K_1 and vitamin K, 2,3-epoxide.* Plasma concentrations of *trans*-vitamin K_1 and vitamin K, 2,3-epoxide were determined by high performance liquid chromatography by the method of Wilson and Park [17].

RESULTS

Pharmacological response to vitamin K in anticoagulated rabbits

The pharmacological response to vitamin K was determined by measuring P.C.A. at various time points in rabbits anticoagulated with a single dose of brodifacoum (10 mg/kg) which is ten times greater than that required to produce maximum antagonism of vitamin K_1 for at least one week [14]. Chronic administration of warfarin (63 mg/kg) at eight-hourly intervals was necessary to produce comparable antagonism of vitamin K_1. After intravenous administration of vitamin K_1 (1 mg/kg) P.C.A. increased sharply and reached a maximum (64 ± 19%) at 3 hr, and then declined at a rate ($t_{\frac{1}{2}}$ = 4.94 ± 0.45 hr)

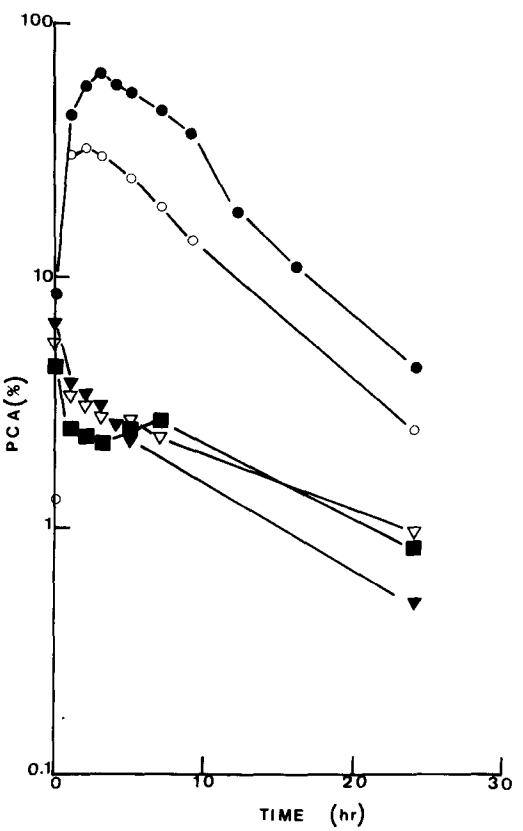

Fig. 1. Prothrombin complex activity vs time in separate groups of rabbits anticoagulated with brodfacoum (10 mg/kg) after intravenous administration of vitamin K_1 (1 mg/kg) (●), vitamin K_2 (1 mg/kg) (○), vitamin K_3 (1 mg/kg) (▼), *cis*-vitamin K_1 (1 mg/kg) (■), and vitamin K_1 2,3-epoxide (1 mg/kg) (▽). Results are presented as means (N = 4).

which indicates maximum inhibition of clotting factor synthesis in the rabbit [14]. The duration of action of molar equivalent doses of vitamin K_1 and related compounds were investigated in separate groups of animals. Vitamin K_2 was less effective than vitamin K_1 and P.C.A. reached a maximum (32.3 ± 2.5%) at 2 hr and then declined, again at a rate corresponding to maximum inhibition of clotting factor synthesis. Using the same test system, we found that intravenous administration of equivalent doses of *cis*-vitamin K_1, vitamin K_3 and vitamin K_1 2,3-epoxide produced no pharmacological response and that in each case P.C.A. continued to decline (Fig. 1).

In keeping with our previous work [14], we found that the duration of pharmacological response to vitamin K_1 is dependent upon dose. Thus after intravenous administration of a larger dose (10 mg/kg) P.C.A. reached a maximum (100.0 ± 30.6%) at 9 hr after which it declined again at a rate (6.13 ± 0.53 hr) which corresponds to complete inhibition of clotting factor synthesis (Fig. 2). Hence the experimental model used in this study provides a sharp pharmacological end point with which to determine the duration of action in vitamin K_1.

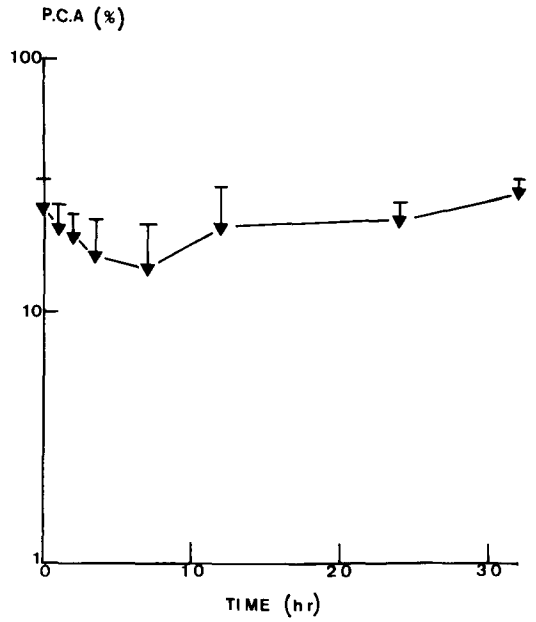

Fig. 3. Prothrombin complex activity vs time in rabbits anticoagulated with 2-chlorovitamin K (10 mg/kg) after intravenous administration of vitamin K_1 (10 mg/kg). Results are presented as means (N = 4) ± S.D.

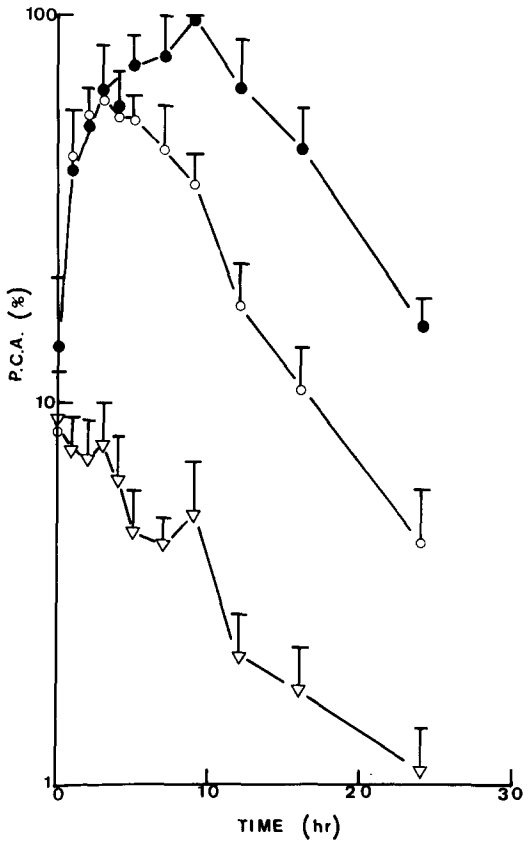

Fig. 2. Prothrombin complex activity vs time in rabbits anticoagulated with brodifacoum (10 mg/kg), after intravenous administration of vitamin K_1 at doses of 1 mg/kg (○) and 10 mg/kg (●) and after 1 mg/kg p.o. (▽). Results are presented as means (N = 4) ± S.D.

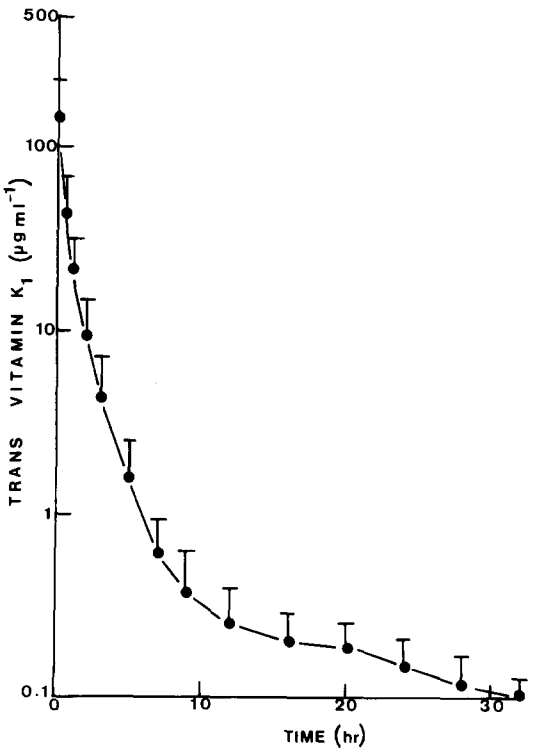

Fig. 4. Plasma concentrations of *trans*-vitamin K_1 vs time in rabbits after intravenous administration of vitamin K_1 (10 mg/kg). Results are presented as means (N = 4) ± S.D.

The effect of route of administration on the pharmacological response to vitamin K_1 is illustrated in Figs. 2 and 7a. Oral administration of a dose (1 mg/kg) which was effective when administered intravenously, did not produce a significant increase in P.C.A. which continued to decline. Administration of a larger (10 mg/kg) dose produced a pharmacological response in only two out of five rabbits tested (Fig. 7a). In these two animals P.C.A. began to rise 1 hr after administration of the vitamin and reached a maximum (32.9% and 51.1%) at between 5 and 7 hr, and then declined.

The pharmacological response to vitamin K_1 (10 mg/kg) in rabbits anticoagulated with 2-chlorovitamin K, which acts by inhibition of the epoxidase/carboxylase system, is shown in Fig. 3. Throughout the time period studied (0–30 hr), there was no significant increase in P.C.A.

Plasma disposition of vitamin K_1 in relation to the pharmacological response

The plasma concentrations of *trans*-vitamin K_1 declined tri-exponentially after intravenous administration of a pharmacological dose (10 mg/kg) in control rabbits (Fig. 4). The correlation coefficients for the regression analysis for each of the three phases were >0.95 in all cases. Up to twelve hours following intravenous administration, plasma concentrations of the vitamin declined steeply and the two initial half-lives, $t_{\frac{1}{2}}\alpha$ (0.35 ± 0.08 hr) and $t_{\frac{1}{2}}\beta$ (1.59 ± 0.46) are similar to those obtained previously using a tracer dose of the vitamin [18]. After 12 hr concentration declined with a half-life $t_{\frac{1}{2}}\gamma = 18.9 \pm 9$ hr. However, the low plasma levels measured during this phase would not have been detected with the analytical techniques used in our previous work [18]. Prior administration of either brodifacoum (10 mg/kg) or 2-chlorovitamin K (10 mg/kg) did not alter the plasma concentration profile of vitamin K_1 up to 28 hr. However, brodifacoum did significantly increase ($P < 0.01$) plasma concentrations of vitamin K_1 2,3-epoxide from 5 hr to 24 hr compared with controls, while 2-chlorovitamin K, in contrast, produced a significant ($P < 0.01$) decrease in vitamin K_1 2,3-epoxide levels from 1 to 7 hr (Fig. 6).

The relationship between the pharmacological response to vitamin K_1 and plasma levels may be obtained directly by reference to Fig. 2 and to Fig. 5 which show data obtained for the same three groups of animals. After intravenous administration of vitamin K_1, we may define duration of action as the time taken to reach maximum P.C.A. since thereafter P.C.A. declines at a rate corresponding to complete inhibition of clotting factor synthesis (Fig.

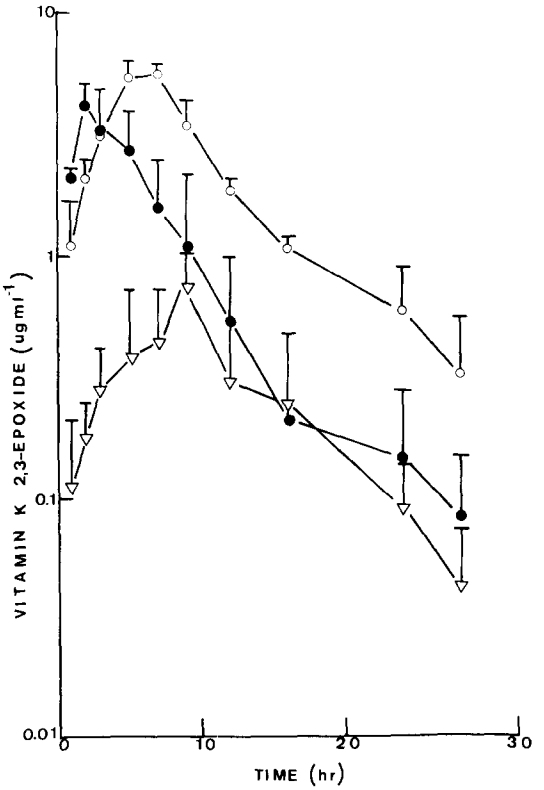

Fig. 6. Plasma concentrations of vitamin K_1 2,3-epoxide vs time after intravenous administration of vitamin K_1 (10 mg/kg) to control rabbits (●) and rabbits anticoagulated with either brodifacoum (10 mg/kg; ○) or chlorovitamin K (10 mg/kg; ▽). Results are presented as means (N = 4) ± S.D.

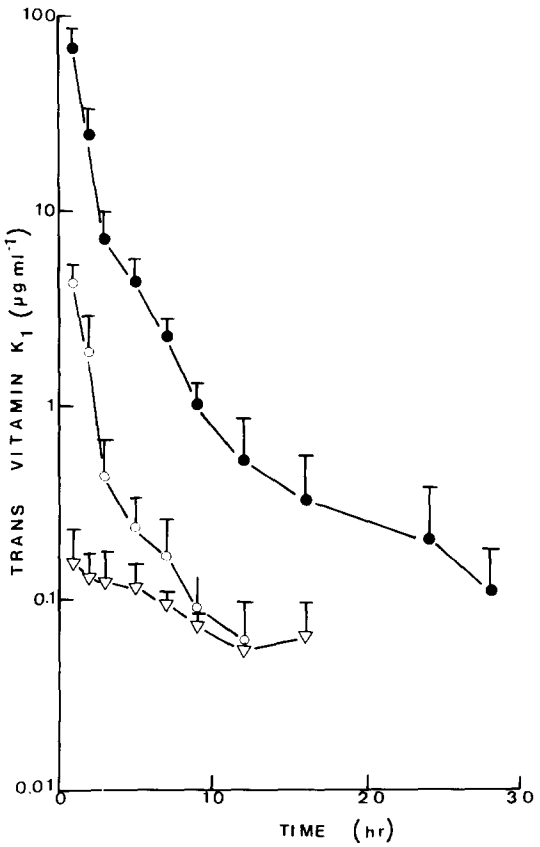

Fig. 5. Plasma concentrations of *trans*-vitamin K_1 vs time in rabbits anticoagulated with brodifacoum (10 mg/kg) after intravenous administration of vitamin K_1 at doses of 1 mg/kg (○) and 10 mg/kg (●) and after 1 mg/kg p.o. (▽). Results are presented as means (N = 4) ± S.D.

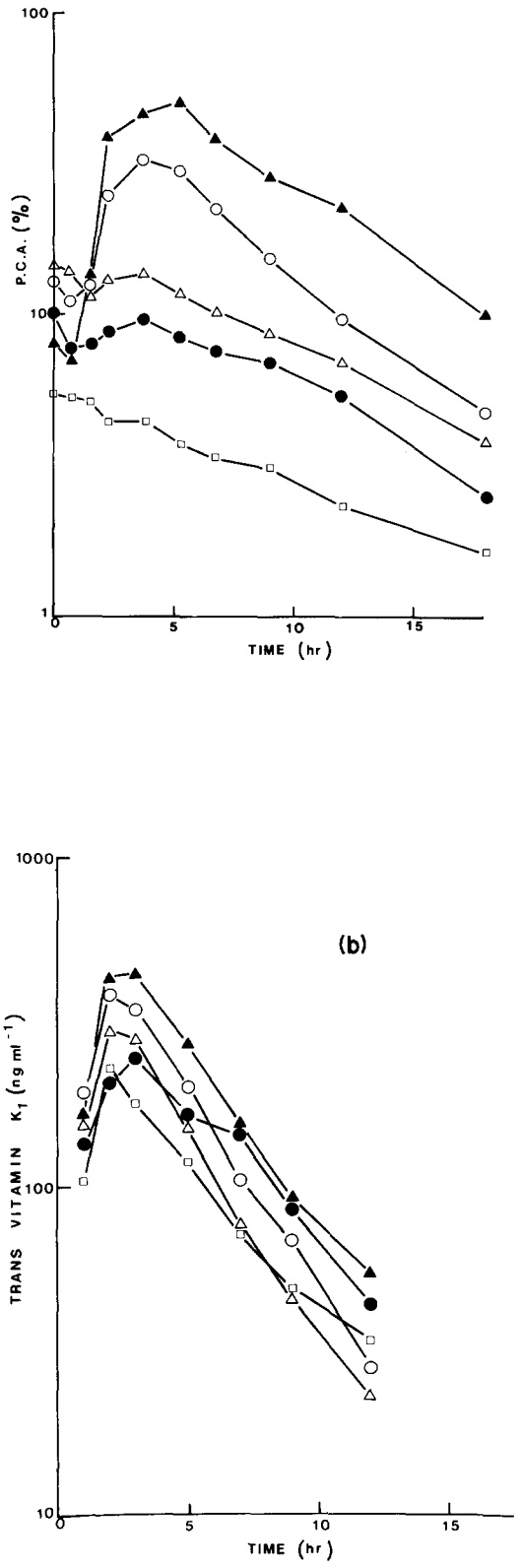

Fig. 7. (a) Prothrombin complex activity vs time in five individual rabbits anticoagulated with brodifacoum (10 mg/kg) after oral administration of vitamin K_1 (10 mg/kg). (b) Plasma concentrations of *trans*-vitamin K_1 vs time in five individual rabbits anticoagulated with brodifacoum (10 mg/kg) after oral administration of vitamin K_1 (10 mg/kg).

2). Thus extrapolation to Fig. 5 indicates that the minimum plasma concentration (3 hr) of vitamin K_1 required to drive clotting factor synthesis after 1 mg/kg is 0.43 ± 0.23 μg/ml while after 10 mg/kg the corresponding plasma concentration (9 hr) is 1.00 ± 0.38 μg/ml. The maximum plasma concentration (0.16 ± 0.07 μg/ml) of vitamin K_1 after oral (1 mg/kg) administration was observed at 1 hr (Fig. 5). After oral administration of a larger dose (10 mg/kg) plasma concentrations peaked at between 2 and 3 hr and covered a range of 0.23–0.44 μg/ml (Fig. 7b). A pharmacological response was only observed (Fig. 7a) in the two animals in which maximum plasma levels of the vitamin were 0.39 and 0.44 μg/ml (Fig. 7b).

DISCUSSION

The nature of the pharmacological response to vitamin K_1 in anticoagulated animals is dependent upon the mechanism of action of the particular anticoagulant. 2-Chlorovitamin K was referred to initially as a direct antagonist of vitamin K_1 [19] and is now thought to act by inhibition of the epoxidase/carboxylase system [16, 20]. In keeping with this hypothesis, we found that vitamin K_1 (10 mg/kg) did not increase P.C.A. in 2-chlorovitamin K anticoagulated rabbits (Fig. 3) and that plasma concentrations of vitamin K_1 2,3-epoxide were reduced in these animals, compared with controls (Fig. 6). In contrast coumarin anticoagulants cannot block the response to high doses of the vitamin (Fig. 2). Thus i.v. administration of vitamin K_1 (10 mg/kg) produced a marked significant increase in P.C.A in rabbits given a dose of brodifacoum which produces maximum antagonism of vitamin K_1 [14]. The pharmacological response to the vitamin was characterised by the sharp rise in P.C.A., followed by a slower rise, and then a decline at a rate which corresponds to complete inhibition of clotting factor synthesis [16]. The initial fast rise probably reflects rapid γ-carboxylation of clotting factor precursors which have accumulated in the liver [21]. The inability of coumarin anticoagulants to completely antagonise vitamin K_1 is consistent with the hypothesis that they act indirectly by inhibition of the epoxide reductase and do not block γ-carboxylation directly [9, 16, 22].

Maximum antagonism of Vitamin K_1 is observed for at least a week after a single 1 mg dose of brodifacoum [14]. Chronic administration of 100 molar equivalents of warfarin was required to produce comparable antagonism. Brodifacoum is therefore a useful research tool for the investigation of vitamin K requirements in animals which lack a functional epoxide reductase. Using brodifacoum-anticoagulated rabbits, we found that the duration of the pharmacological response to vitamin K_2 was less than that observed for an equivalent dose of vitamin K_1 (Fig. 1), while vitamin K_3 produced no stimulation of clotting factor synthesis. Vitamin K_3 has an activity similar to that of vitamin K_1 in the curative chicken test [23]. It was suggested that vitamin K_3 can be regarded as pro-vitamin K_1 because it must be first substituted in the 3-position before it can exert a biological effect. Presumably this process occurs too

slowly to provide the levels of vitamin K_1 required to drive clotting factor synthesis during chronic coumarin anticoagulation. Synthetic vitamin K_1 is a 12:88 mixture of *cis* and *trans* isomers [17]. *Trans*-vitamin K_1 is the natural form of the vitamin while *cis*-vitamin K_1 is thought to be biologically inactive, and is a poor substrate for epoxidation [24]. Accordingly, we found that *cis*-vitamin K_1 was inactive in our test system. Finally, we found that vitamin K_1 2,3-epoxide was ineffective, in accordance with the proposed site of action of coumarin anticoagulants.

The duration of action of vitamin K_1 during coumarin anticoagulation is short in animals and man [12–14]. Thus in the limiting situation of maximum antagonism, large doses of vitamin K_1 (1–10 mg/kg) maintained clotting factor synthesis for only 3–9 hr (Fig. 2). Furthermore oral administration of vitamin K_1 at a dose of 1 mg/kg was ineffective, while only two out of five animals responded to a dose of 10 mg/kg. To explain these findings, we investigated the relationship between the plasma disposition and pharmacological response of the vitamin.

The disposition of vitamin K_1 in plasma has been examined after administration of radiotracer doses in man, dog, rabbit and rat [18, 25–28]. In each species plasma concentrations decline bi-exponentially with a terminal half-life of between 1 and 4 hr. In these studies plasma concentrations of vitamin K_1 were measured for up to 12 hr. In the present study we were able to measure the plasma concentration of vitamin K_1 for up to 32 hr after administration of a high, pharmacological dose (10 mg/kg) to control rabbits. For the first 12 hr, the pharmacokinetic parameters were similar to those reported previously for a more physiological dose (2.17 μg/kg) [18]; suggesting no pronounced dose-dependency in the pharmacokinetics of the vitamin. However, after 12 hr, we discovered a third elimination phase from plasma with a mean half-life of 18.9 ± 9 hr. This phase would not have been detected with the analytical techniques used previously. In accordance with previous work [16, 18], brodifacoum did not alter the plasma concentration–time profile of vitamin K_1, but did increase plasma concentration of vitamin K_1 2,3-epoxide.

Comparison of the pharmacokinetic data in Fig. 5 and the pharmacodynamic data in Fig. 2 indicates that the minimum plasma concentration of vitamin K_1 required for clotting factor synthesis lies in the range 0.4–1.0 μg/ml. However, the precise concentration of vitamin K_1 required to drive clotting factor synthesis can only be determined by direct measurement of the vitamin in the endoplasmic reticulum of hepatocytes. The high plasma concentrations of vitamin K_1 required for clotting factor synthesis in chronic coumarin poisoning contrasts with normal hepatic concentrations of the vitamin (44 ng/g) in the rat [29], and the estimated body pool of *ca.* 100 μg/kg [8, 25]. However, the small pool of vitamin K_1 associated with clotting factor synthesis is normally conserved in the vitamin K_1-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 [30–32]. Thus in the absence of a functional epoxide reductase (i.e. during chronic brodifacoum anticoagulation), recycling of vitamin K_1 does not occur and therefore vitamin K_1 requirements are much greater than normal.

The maximum plasma concentrations of vitamin K_1 after oral administration of a pharmacological dose (1 mg/kg) were less than 300 ng/ml; consequently there was no stimulation of clotting factor synthesis. Vitamin K_1 is absorbed from the proximal portion of the small intestine in an energy dependent saturable process and is then transported via the thoracic lymph duct [33]. This may explain the wide inter-animal variation in plasma concentrations, produced after oral administration of a large (10 mg/kg) dose which in turn may explain the variation in pharmacological response.

In conclusion, we have shown that the brodifacoum-anticoagulated rabbit is a useful animal model for the investigation of factors which determine the pharmacological response to vitamin K during coumarin anticoagulation. Using this experimental system, we found that vitamin K_1 is more effective than vitamin K_2, while synthetic vitamin K_3 and *cis*-vitamin K_1 were ineffective. Plasma concentrations of the order of 0.4–1.0 μg/ml are required to maintain clotting factor synthesis in the limiting situation of maximum coumarin antagonism of the vitamin. This requirement for such high concentrations, taken together with the steep decline in plasma vitamin K_1 concentrations after inravenous administration of a pharmacological dose, provides an explanation for the short duration of the vitamin in such circumstances.

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