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The relationship between inhibition of vitamin K_1 2,3-epoxide reductase and reduction of clotting factor activity with warfarin

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1 The effect of low dose steady state warfarin (0.2 mg and 1 mg daily) on clotting factor activity and vitamin K_1 metabolism was studied in seven healthy volunteers.
2 Steady state plasma warfarin concentrations were 41–99 ng ml^{-1} for the 0.2 mg dose and 157–292 ng ml^{-1} for the 1 mg dose.
3 There was a significant prolongation of the mean prothrombin time (0.9 s) after 1 mg warfarin daily, but no significant change in prothrombin time after 0.2 mg warfarin daily. There was no significant change in individual clotting factor activity (II, VII, IX or X) with either dose of warfarin.
4 Following the administration of a pharmacological dose of vitamin K_1 (10 mg), all seven volunteers had detectable levels of vitamin K_1 2, 3-epoxide with both doses of warfarin ($C_p$max 31–409 ng ml^{-1}).
5 Both the $C_p$max and the AUC for vitamin K_1 2, 3-epoxide were significantly greater on 1 mg of warfarin daily than 0.2 mg daily ($P < 0.01$).
6 The apparent dissociation between inhibition of vitamin K_1 2, 3-epoxide reductase and reduction of clotting factor activity, produced by warfarin, may reflect the insensitivity of functional clotting factor assays to a small reduction in clotting factor concentration.

Keywords vitamin K_1 warfarin clotting factor activity

Introduction

Vitamin K is an essential cofactor for the post ribosomal synthesis of clotting factors II, VII, IX and X (Stenflo & Suttie, 1977). During clotting factor synthesis vitamin K_1 is converted into an inactive metabolite vitamin K_1 2,3-epoxide, which is rapidly reduced back to the vitamin by a microsomal epoxide reductase (Willingham & Matschiner, 1974; Bell, 1978). Cyclic interconversion of vitamin and epoxide is referred to as the vitamin K epoxide cycle. It has been proposed that 4-hydroxycoumarin anticoagulants, such as warfarin, block clotting factor synthesis by inhibition of the regeneration of vitamin K_1 from the inactive vitamin K_1 2, 3-epoxide in plasma (Bell & Matschiner, 1972). Consistent with this hypothesis, it has been shown that administration of coumarin anticoagulants such as warfarin, difenacoum and brodifacoum cause an accumulation of vitamin K_1 2,3-epoxide in plasma, in man and in experimental animals, after administration of either a physiological or pharmacological dose of the vitamin (Shearer *et al.*, 1977; Park *et al.*, 1979; Choonara *et al.*, 1985). Furthermore, the effect of warfarin on vitamin K_1 metabolism is dose dependent (Shearer *et al.*, 1977).

We have previously reported the inhibition of

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vitamin K_1 2,3-epoxide reductase, in the presence of normal clotting factor activity, in two patients accidentally exposed to the long acting coumarin anticoagulants difenacoum and brodifacoum (Park *et al.*, 1986). We decided therefore to study the effect of low dose warfarin on clotting factor activity and vitamin K_1 metabolism to see whether this apparent dissociation is unique to brodifacoum and difenacoum, or a property of all 4-hydroxycoumarin anticoagulants.

Methods

Plan of study

Seven healthy volunteers each took (i) 1 mg, (ii) 0.2 mg racemic warfarin daily for 3 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 local ethics committees. Once steady state warfarin levels had been reached (2 weeks), they received a single intravenous dose of vitamin K_1 (10 mg), diluted in 10 ml 0.9% saline, given over 10 min. They continued to take warfarin for 96 h after the administration of the vitamin K_1.

Venous blood samples were collected prior to the warfarin and at 0, 0.5, 1, 2, 4, 6, 8, 24, 48, 72 and 96 h after the vitamin K_1. Commercially available tablets were used for the 1 mg dose of warfarin, and gelatin capsules were prepared containing 0.2 mg warfarin and lactose.

Analytical methods

Vitamin K_1 (the biologically active *trans*-isomer) and vitamin K_1 2, 3-epoxide concentrations were determined using a normal phase high performance liquid chromatography assay (Wilson & Park, 1983). Plasma warfarin concentrations were determined using a normal phase high performance liquid chromatography assay (Breckenridge *et al.*, 1985). Prothrombin time and the activity of individual clotting factors were measured using established procedures (Hall & Malia, 1984). Protein C and factor II antigens were determined by one dimensional immunoelectrophoresis (Laurell, 1966; Malia *et al.*, 1980). Functional protein C activity was determined using snake venom (*Agkistrodon contortrix contortrix*) (Stocker *et al.*, 1986).

Calculations

A biexponential equation was fitted to the vitamin K_1 plasma concentration *vs* time curve data using a regression analysis programme (Nielsen-Kudsk, 1980). The area under the plasma concentration-time curve (AUC) for vitamin K_1 2, 3-epoxide was determined by the trapezoidal rule up to 8 h in all the volunteers. Statistical analysis was by the paired Student's *t*-test.

Results

The pharmacokinetic values for vitamin K_1 and vitamin K_1 2, 3-epoxide for each individual are given in Table 1. The dose of warfarin had no significant effect on the plasma half-life of vitamin K_1. All seven volunteers had detectable levels of vitamin K_1 2, 3-epoxide with both doses of warfarin. The mean (± s.e. mean) plasma concentration time curve for the epoxide is shown in Figure 1. Both the $C_p$max and the AUC for the epoxide were significantly greater ($P < 0.01$) with the higher dose of warfarin (Table 1 and Figures 2 and 3). The trough plasma concentrations of warfarin are also shown in Table 1, and as expected were significantly greater with the higher dose of warfarin ($P < 0.001$). In one volunteer (PW), trough plasma warfarin concentrations were below the limit of sensitivity with the low dose of warfarin.

The pharmacodynamic effect of warfarin on prothrombin time and individual clotting factor activity is shown in Table 2. The higher dose of warfarin caused a small but significant ($P <$ 0.05) prolongation of the mean prothrombin time (0.9 s). There was however a clear prolongation of the prothrombin time (2.5 s) in only one volunteer (IC). This individual also had a clear reduction in individual clotting factor activity on 1 mg warfarin daily. No other volunteer had a consistent fall in clotting factor activity on 1 mg warfarin daily. The low dose of warfarin had no significant effect on either prothrombin time or clotting factor activity ($P > 0.05$). In all seven individuals the prothrombin time returned to its initial value for at least one time point after vitamin K_1. There was no significant reduction in the individual clotting factors when considered separately.

In three individuals, protein C was also measured (Table 3). The protein C: factor II antigen ratio was in the normal range for all the volunteers, thus excluding congenital protein C deficiency. There was no change in protein C antigen or the protein C: factor II antigen ratio after the administration of vitamin K_1. There was however a fall in protein C activity in one volunteer (IC) on the higher dose of warfarin. Protein C activity returned to normal for a period of 24 h, after the administration of vitamin K_1 in this volunteer.

Table 1 Pharmacokinetics of vitamin K_1 and vitamin K_1 2, 3-epoxide in healthy volunteers at steady state warfarin (1 mg or 0.2 mg daily), after a single dose of vitamin K_1 (10 mg i.v.)

Volunteer		*Vitamin K_1* $t_{1/2}$ *(h)*	*Vitamin K_1 2, 3-epoxide* C_pmax *(ng ml^{-1})*	*AUC (ng ml^{-1} h)*	*Plasma warfarin (ng ml^{-1})*
IC	1 mg	2.01	212	1367	269
	0.2 mg	1.37	205	1149	99
PW	1 mg	1.33	207	1215	180
	0.2 mg	1.06	68	277	ND
BH	1 mg	3.14	248	1537	157
	0.2 mg	1.49	70	329	72
MT	1 mg	3.46	176	1050	242
	0.2 mg	1.61	49	153	92
GP	1 mg	1.66	342	1958	215
	0.2 mg	2.32	110	537	70
JB	1 mg	2.23	302	1596	260
	0.2 mg	1.78	31	113	41
JF	1 mg	2.21	409	2221	292
	0.2 mg	2.07	126	534	47
Mean	1 mg	2.29	271	1563	231
	0.2 mg	1.67	94	442	60

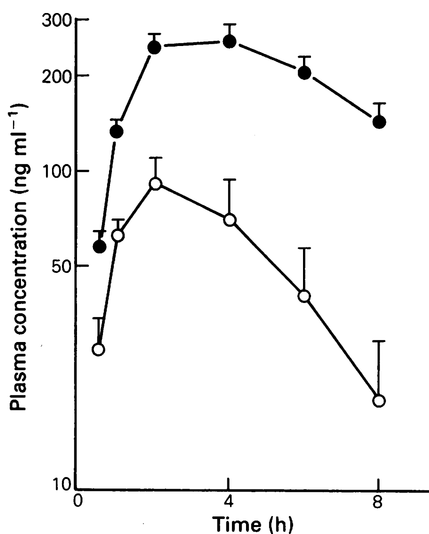

Figure 1 Mean plasma concentrations (± s.e. mean) of vitamin K_1 2, 3-epoxide in healthy volunteers on steady state warfarin (0.2 mg (○) or 1 mg (●) daily), after the administration of a single dose of vitamin K_1 (10 mg i.v.).

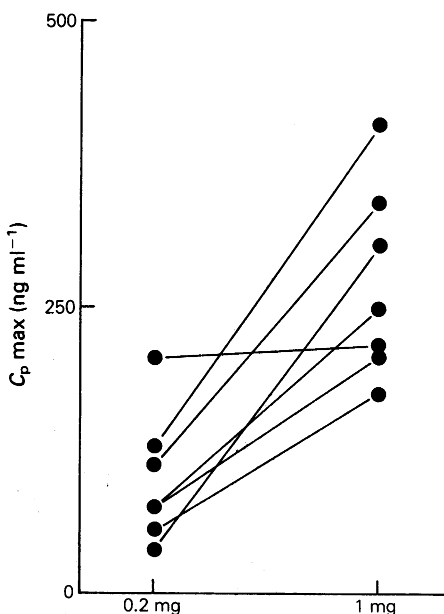

Figure 2 C_p max for vitamin K_1 2, 3-epoxide in healthy volunteers on steady state warfarin (0.2 mg or 1 mg daily), after the administration of a single dose of vitamin K_1 (10 mg i.v.).

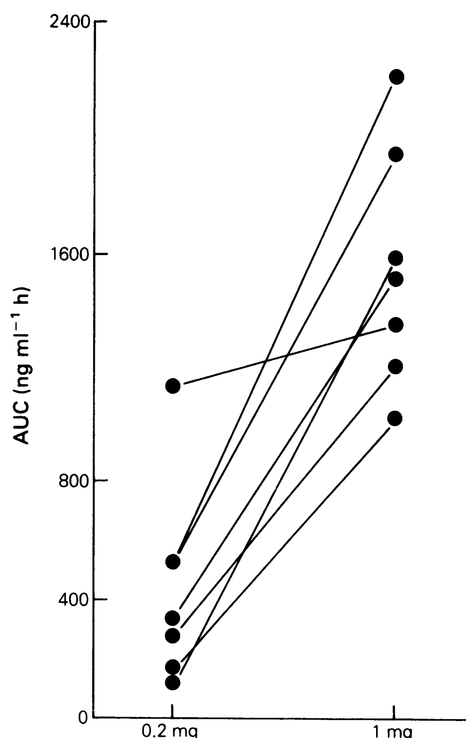

Figure 3 AUC for vitamin K_1 2,3-epoxide in healthy volunteers on steady state warfarin (0.2 mg or 1 mg daily), after the administration of a single dose of vitamin K_1 (10 mg i.v.).

Discussion

The detection of vitamin K_1 2, 3-epoxide in all seven volunteers on both doses of warfarin confirms that warfarin inhibits the enzyme vitamin K_1 epoxide reductase. Vitamin K_1 2, 3-epoxide is not detectable in volunteers after the administration of vitamin K_1 in the absence of 4-hydroxycoumarins (Park *et al.*, 1984). There was a clear dose dependent inhibition of epoxide reductase, levels of the epoxide (both $C_p$max and the AUC) being greater with the higher dose of warfarin. There was however considerable inter-individual variation in the levels of epoxide. The findings are consistent with previous work (Shearer *et al.*, 1977) which showed a direct relationship between the log of the plasma warfarin concentration and the $C_p$max of [^{3}H]-vitamin K_1 2, 3-epoxide, after administration of a lower dose of the vitamin.

The pharmacodynamic effect of warfarin was monitored by measurement of prothrombin time and the individual vitamin K_1 dependent clotting factors. There was a small but significant prolongation of the prothrombin time with 1 mg warfarin daily, but no measurable pharmacodynamic effect with 0.2 mg warfarin daily. This confirms the greater sensitivity of vitamin K_1 2, 3-epoxide as a marker of the presence of 4-hydroxycoumarins than prothrombin time or individual clotting factor activity, as previously suggested (Park *et al.*, 1986). There is a direct linear relationship between prothrombin time and the reciprocal of prothrombin complex activity (PCA) (Biggs & Denson, 1967). Therefore the prothrombin time is sensitive to minor changes in PCA when the % PCA is low. However when the % PCA is high, the prothrombin time is relatively insensitive to minor changes in PCA.

The findings raise important questions about the mode of action of warfarin. The inhibition of vitamin K_1 epoxide reductase without any change in clotting factor activity may be due to one of three reasons:

(i) It is possible that warfarin does not act directly by the inhibition of vitamin K_1 epoxide reductase. That is, warfarin inhibits other enzymes involved in vitamin K_1 metabolism as well as vitamin K_1 epoxide reductase. The inhibition of these other enzymes may play a role in the inhibition of γ-carboxylation of vitamin K_1 dependent clotting factors. This hypothesis is supported by *in vitro* studies which suggest a possible effect on vitamin K_1 quinone reductase (Fasco *et al.*, 1982; Preusch & Suttie, 1984).

(ii) Another possibility is that there is a threshold for inhibition of vitamin K_1 epoxide reductase, below which one does not get a change in clotting factor synthesis. The degree of enzyme inhibition required in order for a drug to produce a detectable pharmacodynamic effect is probably related to the amount of enzyme present within a tissue. For example, due to the presence of an excess of carbonic anhydrase in the kidney, more than 99% enzyme inhibition is required for acetazolamide to produce a pharmacological effect (Mudge, 1980).

(iii) It is possible that inhibition of vitamin K_1 epoxide reductase and inhibition of γ-carboxylation of vitamin K_1 dependent clotting factors are directly related. The demonstration of an apparent dissociation between the two processes could be due to the relative insensitivity of the measurement of prothrombin time and individual clotting factor activity with regard to changes in circulating clotting factor protein. To confirm a biochemical dissociation between the two processes, would require the measurement of levels of γ-carboxylated clotting factors. It has recently become possible to measure γ-carboxylated prothrombin (Blanchard *et al.*, 1983)

Table 2 Effect of steady state warfarin (1 mg or 0.2 mg daily) on clotting factor activity and prothrombin time in healthy volunteers, prior to the administration of vitamin K_1

Volunteer		*II*	*VII*	*IX*	*X*	*PT (s)*		*II*	*VII*	*IX*	*X*	*PT (s)*
IC	Pre	86	94	92	76	13.0	Pre	100	88	88	74	13.0
	1 mg	70	73	71	49	15.5	0.2 mg	98	92	94	81	13.0
PW	Pre	60	74	80	70	13.5	Pre	68	74	78	70	13.0
	1 mg	72	75	91	75	14.0	0.2 mg	73	70	81	87	13.0
BH	Pre	105	73	95	88	13.0	Pre	96	80	100	87	13.5
	1 mg	107	84	100	94	13.8	0.2 mg	105	82	91	84	13.8
MT	Pre	75	80	105	70	13.0	Pre	92	110	108	88	13.0
	1 mg	81	97	108	66	13.8	0.2 mg	75	112	102	67	13.0
GP	Pre	100	108	72	84	12.5	Pre	100	112	105	112	12.5
	1 mg	103	102	80	98	12.5	0.2 mg	105	112	89	107	13.0
JB	Pre	84	134	100	83	13.0	Pre	82	94	70	86	13.5
	1 mg	115	100	123	61	13.8	0.2 mg	74	108	75	88	14.1
JF	Pre	80	100	76	88	13.0	Pre	74	100	80	105	12.8
	1 mg	75	78	76	72	13.7	0.2 mg	75	101	73	106	13.0
Mean	Pre	84	95	89	80	13.0	Pre	87	94	90	89	13.0
	1 mg	89	87	93	74	13.9	0.2 mg	86	97	86	89	13.2

Pre = Prior to warfarin.
1 mg = On steady state warfarin (1 mg daily), prior to vitamin K_1.
0.2 mg = On steady state warfarin (0.2 mg daily), prior to vitamin K_1.
The activities of the individual clotting factors are expressed as a percentage of the normal value obtained using pooled serum. The normal range for the factors are as follows: II (60–167%), VII (58–162%), IX (56–175%), X (58–124%).

Table 3 Effect of steady state warfarin (0.2 mg or 1 mg daily) on factor II antigen and protein C in healthy volunteers, prior to the administration of vitamin K_1

Volunteer		*II antigen*	*Protein C antigen*	*Protein C functional*	*Protein C antigen / II antigen*	*Protein C functional / Protein C antigen*
IC	Pre	86	100	140	1.16	1.40
	0.2 mg	81	103	137	1.05	1.33
	Pre	76	96	140	1.26	1.40
	1 mg	91	95	71	1.04	0.75
BH	Pre	115	96	112	0.83	1.17
	0.2 mg	125	100	98	0.80	0.98
	Pre	125	118	116	0.94	0.98
	1 mg	126	102	103	0.81	1.01
GP	Pre	88	100	100	1.14	1.00
	0.2 mg	78	97	94	1.24	0.97
	Pre	108	94	82	0.87	0.87
	1 mg	81	91	105	1.12	1.15

Pre = Prior to warfarin.
0.2 mg = On steady state warfarin (0.2 mg daily), prior to vitamin K_1.
1 mg = On steady state warfarin (1 mg daily), prior to vitamin K_1.
Factor II antigen and protein C (functional and antigen) are expressed as a percentage of the normal value obtained using pooled serum.

and this appears to be a more sensitive marker of the functional aspects of coagulation than the prothrombin time (Furie *et al.*, 1984). Whether the test is sensitive enough to detect minor changes in the levels of γ-carboxylated prothrombin is not certain.

The measurement of individual clotting factor activity was not as sensitive as prothrombin time. There was however, considerable intra-individual variation in clotting factor activity and as the assay involves a modification of the one stage prothrombin time (for factors II, VII and X), the slope of the assay was less steep than that for the prothrombin time. A flatter slope results in a loss of discrimination within the assay (Hall & Malia, 1984) and therefore is less likely to detect minor changes in clotting factor activities.

There was no change in the protein C antigen when expressed either alone or in relation to factor II antigen in any of the three individuals studied with either dose of warfarin. Previous studies have all shown a fall in plasma concentration of protein C antigen in the presence of warfarin (Griffin *et al.*, 1981; Bern *et al.*, 1984; Epstein *et al.*, 1984; Vigano *et al.*, 1984). There was however a marked fall in functional protein C activity with a higher dose of warfarin in the one volunteer who also showed a fall in clotting factor activity (IC). The results are consistent with recent work (Mikami & Tuddenham, 1986) which suggests there is a greater fall in functional protein C activity than in protein C antigen in the presence of warfarin. The results therefore suggest that the functional protein C assay is more sensitive to minor changes than the antigen assay. There did however appear to be a dissociation between the inhibition of γ-carboxylation of protein C and the inhibition of vitamin K_1 2, 3-epoxide reductase with the lower dose of warfarin.

An apparent dissociation between the inhibition of vitamin K_1 epoxide reductase and clotting factor activity is observed in all seven healthy volunteers by the use of a low dose (0.2 mg) of warfarin at steady state. The greater sensitivity of the detection of vitamin K_1 2, 3-epoxide after the administration of a pharmacological dose of vitamin K_1 than the measurement of either prothrombin time or individual clotting factor activity as a marker of exposure to 4-hydroxycoumarins is confirmed. This greater sensitivity is applicable to warfarin as well as the more potent 4-hydroxycoumarins, bordifacoum and difenacoum. The lack of sensitivity of prothrombin time in this respect, suggests that direct measurement of (γ-carboxylated) prothrombin might be useful in the management of patients who appear resistant to anticoagulants and to monitor accidental exposure to anticoagulants.

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