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Comparative Pharmacokinetics of Coumarin Anticoagulants X: Relationship between Distribution, Elimination, and Anticoagulant Action of Warfarin

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Abstract □ The biological half-life of warfarin in a group of male Sprague–Dawley rats varied from 5 to 28 hr, and the apparent volume of distribution varied from 102 to 320 ml/kg body weight. There was a strong and statistically highly significant positive correlation between the elimination rate constant and the apparent volume of distribution of warfarin in individual rats. There was no relationship between the elimination rate constant for warfarin and either the normal (prewarfarin) prothrombin complex activity or the rate constant for decline of this activity when the synthesis of vitamin K-dependent clotting factors was blocked. The concentration of warfarin in plasma at which the synthesis rate of prothrombin complex activity was inhibited by 50% varied from 0.05 to 1.37 μg/ml and showed a strong and highly statistically significant positive correlation with the biological half-life of warfarin in individual animals. These results demonstrate a pronounced association among the apparent volume of distribution, the elimination rate constant, and the plasma concentration–anticoagulant effect relationship for warfarin which may have to be considered in the design of clinical dosage regimens.

Keyphrases □ Anticoagulants, coumarin—comparative pharmacokinetics of warfarin, relationships between distribution, elimination, and activity, biological half-life, volume of distribution □ Coumarin anticoagulants—comparative pharmacokinetics of warfarin, relationships between distribution, elimination, and activity, biological half-life, volume of distribution □ Warfarin—comparative pharmacokinetics, relationships between distribution, elimination, and activity, biological half-life, volume of distribution □ Pharmacokinetics—warfarin distribution, elimination, and activity

Warfarin is probably the most widely used oral anticoagulant. Its biological half-life varies markedly between subjects. For example, the biological half-life of warfarin in 20 healthy adult human subjects was reported to range from 15 to 80 hr (1, 2), and there are case reports of patients with a warfarin half-life of only 5.5 and 6.1 hr (3, 4). In a study (5) of warfarin elimination by mongrel dogs, half-lives of 21–48 hr were found. In random-bred Sprague–Dawley rats, warfarin half-lives from <5 to 70 hr in males and from 10 to 90 hr in females have been observed (6).

It is generally believed that intersubject differences in pharmacological response to a particular dosage regimen of a drug due to differences in the rate of elimination of that drug may be overcome by adjusting the dosage regimen to attain a given plasma concentration, *i.e.*, the therapeutic concentration. This assumes, for example, that individuals who differ with respect to the half-life for warfarin elimination nevertheless respond equally (at least in the absence of certain other drugs and diseases) to a particular concentration of that drug in the plasma[1]. This assumption would be incorrect if the differences in the elimination kinetics of warfarin are caused by, or associated with, differences in the distribution of that drug in the body. In this study, the apparent volume of distribution, the biological half-life, and the plasma concentration–anticoagulant effect curve for warfarin in individual rats were determined and the relationships between these characteristics were examined.

EXPERIMENTAL

Male Sprague–Dawley rats[2], weighing 305–400 g, were used. They had unrestricted access to food[3] and water before and during the experiments. The animals received 0.6 mg ^{14}C-warfarin/kg body weight ip. Blood samples (0.36 ml) were obtained repeatedly from the tail artery (9) for up to 96 hr after drug administration. Each blood sample was mixed with 0.04 ml sodium oxalate

[1] There are rare instances of hereditary resistance to warfarin and other coumarin anticoagulants, but the distribution and elimination of warfarin in individuals so affected are entirely normal (7, 8).

[2] Blue Spruce Farms, Altamont, N.Y.

[3] Charles River formula 4RF.

Table I—Effect of Different Separation Methods on the Assay of Warfarin in Plasma of Rats after Injection of a Single Dose of ^{14}C-Warfarin[a]

Rat	Time after Injection, hr	Total Radioactivity, μg Apparent Warfarin/ml	Concentration in Plasma, μg/ml					
			O'Reilly Method		Lewis Method		O'Reilly and Lewis Methods Combined	
			Spec.[b]	Scint.[c]	Spec.	Scint.	Spec.	Scint.
A	21.0	4.78	3.31	3.33	3.36	3.56	3.30	3.46
B	24.2	16.4	15.8	15.0	14.2	13.5	15.7	16.3
C	12.0	23.9	21.0	19.6	19.4	19.8	21.4	22.4
D	7.2	36.7	34.6	32.7	35.0	35.6	33.6	33.2

[a] Dose of 12 mg/kg body weight intraperitoneally, including 3.5 μCi ^{14}C-warfarin per rat. [b] Assayed by spectrophotometry. [c] Assayed by scintillation spectrometry.

solution (0.1 *M* in 0.7% sodium chloride), and the hematocrit and prothrombin complex activity (PCA, expressed as percent of normal) were determined as described by Wingard and Levy (9).

The synthesis rate (R_{syn}) and apparent first-order degradation rate constant (k_d) for the PCA were calculated by a previously developed method (10). The k_d was determined following injection of sodium warfarin, 12 mg/kg ip, about 1 month after the study with ^{14}C-warfarin. The biological half-life and apparent first-order elimination rate constant for warfarin were determined from the least-squares slope of plots of the logarithm of the warfarin concentrations in the plasma *versus* time. The apparent volume of distribution was calculated by dividing the injected dose by the extrapolated zero-time plasma warfarin concentration ($V_{intercept}$) or by the product of the elimination rate constant and the area under the plasma concentration *versus* time curve starting at zero concentration at zero time (V_{area}).

The injection solution was prepared within 2 hr before use by dissolving 2.17 mg ^{14}C-warfarin[4] (23 μCi/mg) in about 0.2 ml 0.001 *M* sodium hydroxide solution and immediately adding sufficient normal saline solution to yield a total volume of 10 ml. An aliquot of this solution showed only one peak by radiochromatogram scanning and yielded a concentration of 101% of theory by radioactivity assay.

Warfarin in the plasma was separated from its metabolites by TLC (11), and the eluted warfarin was assayed by scintillation counting. For this purpose, 0.1 ml of plasma was acidified with 0.5 ml 0.3 *N* hydrochloric acid and extracted with 5 ml ethylene dichloride. The phases were separated by centrifuging after 15–20 min of shaking; 4 ml of the organic phase was transferred to a 5-ml capacity vial[5] and evaporated under nitrogen (no heat).

Residual solid material on the walls of the vial was washed to the bottom with acetone and the solvent was evaporated again under nitrogen; this washing and evaporation procedure was repeated twice. The drug in the vial was then dissolved in 30–50 μl acetone and the solution was applied to a TLC sheet. The vial was washed three times with 30-μl portions of acetone and these were also applied to the TLC sheet. During all these procedures, care was taken to protect the drug from light. Chromatography was carried out on silica gel with fluorescent indicator[6] with ethylene dichloride–acetone (9:1). The ^{14}C-warfarin spot was located by a radiochromatogram scanner[7] or was visualized under UV light, using the spot obtained from a solution of warfarin in acetone as a reference. The portion of the chromatogram with the warfarin spot was cut out and dropped into a vial containing 15 ml scintillation fluid (100 g naphthalene, 7 g 2,5-diphenyloxazole, 0.3 g 1,4-bis[2-(5-phenyloxazolyl)]benzene, and 1,4-dioxane to yield 1 liter of solution). Scintillation counting[8] was carried out at room temperature with an internal standard. The counting efficiency averaged 89%. The recovery of warfarin from plasma samples containing 0.05–5.0 μg/ml was 89.7 ± 2.7% (mean ± *SD*, *n* = 14) and showed no dependence on concentration.

In an experiment to compare the results of plasma warfarin assays by the TLC method of Lewis *et al.* (11) with those of the

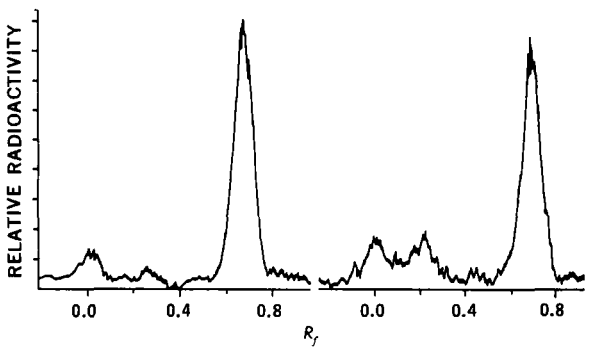

Figure 1—*Radiochromatogram scans of a plasma sample obtained from a rat 21 hr after intraperitoneal injection of ^{14}C-warfarin, 12 mg/kg body weight (Rat A in Table I). The scan on the right was obtained from a thin-layer chromatogram prepared by the method of Lewis* et al. *(11). The scan on the left was obtained by first extracting the plasma sample by the method of O'Reilly* et al. *(12) and then chromatographing the extract by the method of Lewis* et al. *The highest peak in each scan represents the warfarin spot.*

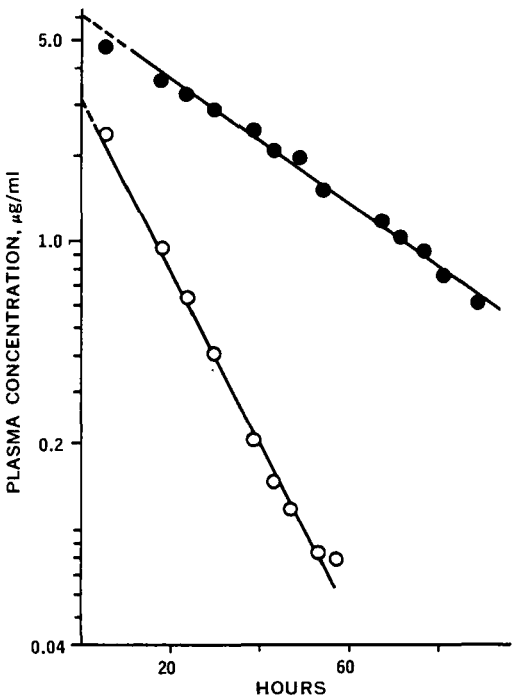

Figure 2—*Warfarin concentration in the plasma of two rats as a function of time after intraperitoneal injection of ^{14}C-warfarin, 0.6 mg/kg body weight. Key: ●, Rat 2; and ○, Rat 5.*

[4] 3-[α-Acetonyl(benzyl-α-^{14}C)]-4-hydroxycoumarin, Amersham-Searle Corp., Arlington Heights, Ill.
[5] Reacti-Vial, Pierce Chemical Co., Rockford, Ill.
[6] Silica gel F-254 TLC sheets, EM Laboratories, Inc., Elmsford, N.Y.
[7] Packard model 7201.
[8] Packard Tri-Carb liquid scintillation counter, model 2002.

Table II—Biological Half-Life, Estimated Apparent Volume of Distribution, and Body Clearance of Warfarin in Rats[a]

Rat	Half-Life, hr	Apparent Volume of Distribution, ml/kg		Total Clearance[d], ml/(kg hr)
		$V_{intercept}$[b]	V_{area}[c]	
1	28.1	104	115	2.83
2	27.7	98	104	2.60
3	26.6	122	133	3.46
4	20.8	94	102	3.40
5	10.1	188	206	14.1
6	8.0	283	320	27.7
7	6.1	248	304	34.5
8	5.1	314	317	43.0

[a] With 0.6 mg warfarin/kg body weight intraperitoneally. [b] Dose divided by the extrapolated zero-time plasma concentration. [c] Dose divided by the product of elimination rate constant and area under the plasma concentration–time curve. [d] Product of V_{area} and elimination rate constant.

spectrophotometric method of O'Reilly *et al.* (12), four rats received warfarin[9], 12 mg/kg ip, and ^{14}C-warfarin, about 3.5 μCi/rat. The four animals were exsanguinated at 7, 12, 21, and 24 hr, respectively. Sufficient blood was obtained from the aorta to yield 4–6 ml plasma, and 0.5-ml portions of each plasma sample were analyzed by both methods. To determine the specificity of the spectrophotometric method, the ethylene dichloride extract of an acidified plasma sample was washed with pH 7.25 phosphate buffer, and this extract was analyzed by the TLC procedure described in the preceding paragraph. The amount of warfarin eluted from the TLC sheet was determined both spectrophotometrically (using microcells) and by scintillation counting.

RESULTS

This investigation required a sensitive and specific method of analysis of warfarin in only 0.1 ml of plasma. The necessary sensitivity was achieved by use of ^{14}C-warfarin. The extraction method of O'Reilly *et al.* (12), the TLC separation method of Lewis *et al.* (11), and a combination of the two methods were evaluated as means of achieving assay specificity. All three methods yielded essentially similar results (Table I). The extraction method of O'Reilly *et al.* (12) separated most, but not all, of the metabolites from warfarin itself (Fig. 1). The more complete separation of metabolites achieved by the Lewis *et al.* (11) method can be important after the concentrations of warfarin have declined by more than one order of magnitude, *i.e.*, when metabolite concentrations may contribute significantly to the total radioactivity in the plasma. Therefore, this TLC method was employed in the subsequent experiments.

The concentration of warfarin in the plasma of individual animals decreased exponentially with time after injection (Fig. 2). Warfarin half-lives ranged from 5.1 to 28.1 hr (Table II). The volumes of distribution ranged from 94 to 314 ml/kg for $V_{intercept}$ and from 102 to 320 ml/kg for V_{area}. There was a strong and

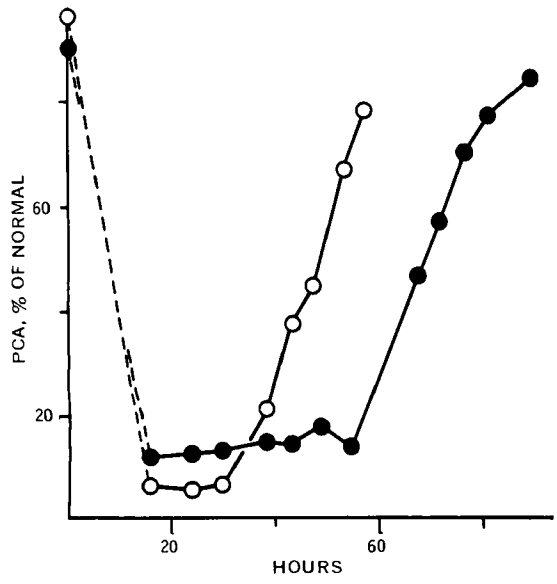

Figure 3—*PCA in the plasma of two rats, one a slow eliminator (●, Rat 2) and the other (○, Rat 5) a rapid eliminator of warfarin, as a function of time after intraperitoneal injection of ^{14}C-warfarin, 0.6 mg/kg body weight.*

[9] Endo Laboratories, Garden City, N.Y.

Table III—Relationship between Biological Half-Life and Duration of Hypoprothrombinemic Effect of Warfarin in Rats

Rat	Half-Life, hr	Time Required for Prothrombin Complex Activity to Return to 60% of Normal, hr
1	28.1	86[a]
2	27.7	72
3	26.6	84
4	20.8	81
5	10.1	50
6	8.0	36
7	6.1	43
8	5.1	34

[a] By extrapolation.

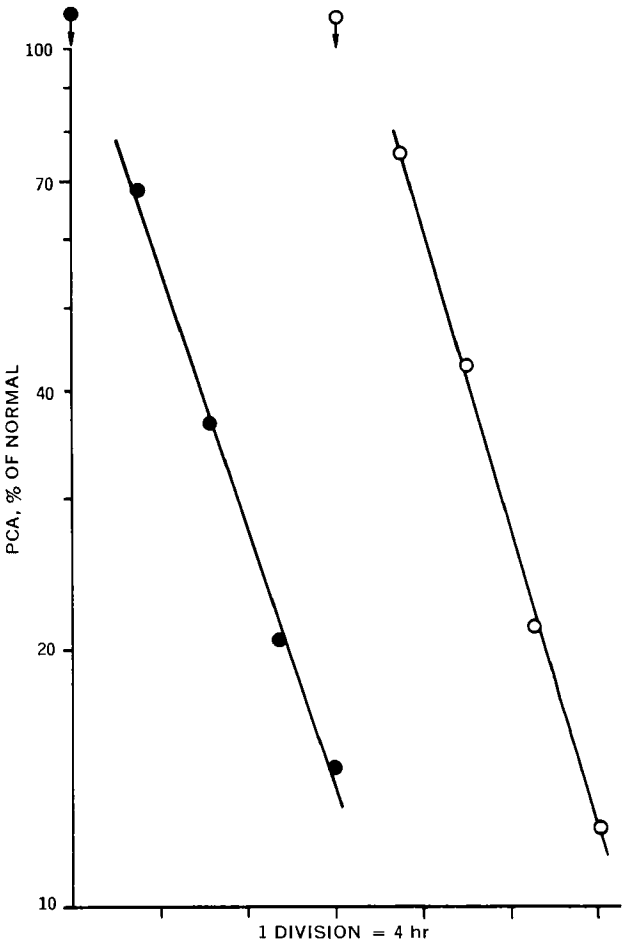

Figure 4—*Decrease in PCA as a function of time after blockade of the synthesis of vitamin K-dependent clotting factors by intraperitoneal injection of sodium warfarin, 12 mg/kg body weight. The arrows indicate the time of injection. Key: ●, Rat 2; and ○, Rat 5.*

Table IV—Normal Prothrombin Complex Activity (P^0), Rate Constant for Decline of Prothrombin Complex Activity (k_d), and Normal Synthesis Rate of Prothrombin Complex Activity (R^0_{syn}) in Rats

Rat	P^0, %	k_d, day^{-1}	R^0_{syn}, % day^{-1}
1	78.6	4.42	347
2	89.6	4.23	379
3	91.9	4.26	392
4	88.1	4.76	419
5	95.9	4.83	463
6	88.1	4.64	409
7	91.9	—[a]	—[a]
8	91.9	4.60	423
Mean	89.5	4.53	408
SD	5.1	0.24	31

[a] Rat 7 was ill at the time of this experiment.

highly statistically significant ($p < 0.001$) positive correlation between the apparent first-order rate constant for warfarin elimination (0.693/half-life) and the apparent volume of distribution of warfarin ($V_{intercept}$ or V_{area}) in individual rats.

The PCA in the plasma decreased rapidly after warfarin injection and eventually returned to normal (Fig. 3). The duration of this effect, as reflected by the time required for PCA to return to 60% of normal, increased with increasing warfarin half-life (Table III). The correlation between warfarin half-life and duration of anticoagulant effect was strong ($r = 0.946$) and highly statistically significant ($p < 0.001$). The prewarfarin PCA (P^0) in the eight rats averaged 89.5% [based on a standard curve obtained previously by determining the clotting time of serial dilutions of pooled plasma from six normal rats, with the PCA of the undiluted plasma defined as 100% of normal (9)]. The rate constant for decline of PCA (k_d) was determined from the slope of a plot (Fig. 4) of log PCA *versus* time after injection of a large dose of warfarin (*i.e.*, a dose sufficient to block the synthesis of vitamin K-dependent clotting factors) and showed very little intersubject variation (Table IV). There was no statistically significant correlation between the elimination rate constant for warfarin and either P^0, k_d, or the normal (prewarfarin) synthesis rate of PCA (R^0_{syn}).

There was an essentially linear relationship between the pharmacological effect of warfarin (expressed as the R_{syn} relative to R^0_{syn}) and the logarithm of the warfarin concentration in plasma at a given time (Fig. 5). Six of the eight animals yielded data that permitted the determination of the warfarin plasma concentration at which R_{syn} = 50% of R^0_{syn}. This concentration varied widely, from 0.052 to 1.37 μg/ml (Table V). A similar wide range was observed in the total radioactivity (reflecting the concentration of both warfarin and its metabolites) at this level of pharmacological activity. There was a strong ($r = 0.963$) and highly statistically significant ($p < 0.001$) correlation between the half-life of warfarin and the warfarin plasma concentration at which R_{syn} = 50% R^0_{syn} in individual rats. There was also a strong relationship between the half-life of warfarin and the concentration of warfarin metabolites (total radioactivity minus warfarin radioactivity) in plasma at the time when R_{syn} = 50% R^0_{syn} ($r = 0.949$, $p < 0.005$). However, it is unlikely that warfarin metabolites contribute significantly to the anticoagulant activity in rats. Of the six metabolites of warfarin identified in rats, only one (4′-hydroxywarfarin) is active, and it has only one-fourth the activity of warfarin itself (13).

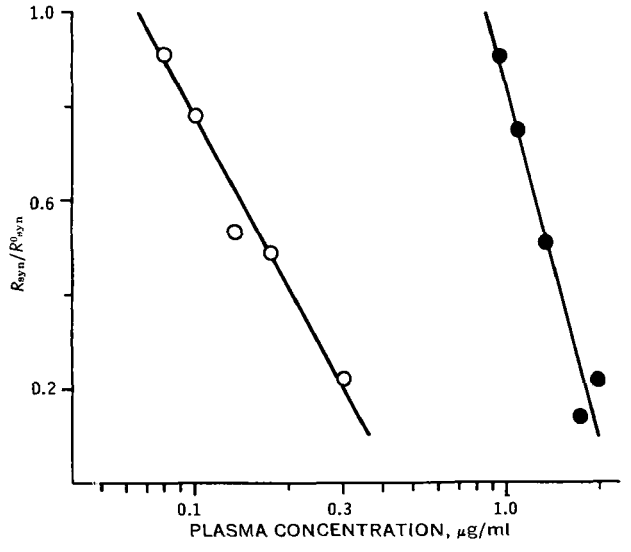

Figure 5—*Relationship between the relative synthesis rate of PCA* (R_{syn}/R^0_{syn}) *and the concentration of warfarin in the plasma of a slow eliminator* (●, *Rat 2*) *and a rapid eliminator* (○, *Rat 5*) *of warfarin after intraperitoneal injection of* ^{14}*C-warfarin, 0.6 mg/kg body weight.*

Table V—Relationship between Biological Half-Life of Warfarin and Plasma Concentration of Warfarin at which the Synthesis Rate of Prothrombin Complex Activity is Decreased by 50%

Rat[a]	Half-Life, hr	Warfarin Concentration in Plasma when R_{syn} = 50% R^0_{syn}, μg/ml[b]	Total Radioactivity in Plasma when R_{syn} = 50% R^0_{syn}, μg Apparent Warfarin/ml
2	27.7	1.37	1.48
3	26.6	0.88	0.98
4	20.8	0.78	0.84
5	10.1	0.17	0.20
6	8.0	0.16	0.20
8	5.1	0.052	0.084

[a] Timing of blood sampling was inadequate for determination of R_{syn} = 50% R^0_{syn} in Rats 1 and 7. [b] Obtained by interpolation from plots such as Fig. 5.

DISCUSSION

The results of these studies show a pronounced relationship between the distribution and elimination of warfarin in rats. This result is consistent with the results of previously reported studies on the elimination of another coumarin anticoagulant, dicumarol, by isolated perfused rat livers (14) and intact rats (15). Intersubject differences in the biological half-life of warfarin can also reflect differences in the activity of drug-metabolizing enzyme system(s), particularly due to enzyme induction, but preliminary data from ongoing experiments indicate that enzyme induction does not cause a change in the apparent volume of distribution of warfarin. It remains to be determined to what extent the activity of the warfarin-metabolizing enzyme system(s) and distributional factors each account for intersubject differences in the elimination of warfarin in humans.

An evaluation of published data on 14 healthy human subjects (1) suggests a possible relationship between the elimination rate constant and the apparent volume of distribution of warfarin in female subjects (four of five in rank order) but there is no such indication for the male subjects. However, one patient with an unusually short half-life of warfarin (6.1 hr) had an apparent volume of distribution of 14.8% of body weight (3), larger than that for 14 normal subjects (1) which ranged from 5.7 to 12.4% of body weight (by calculation of the authors) and larger than the apparent volumes reported by others (16) for nine experiments on six normal subjects (mean 11.4, range 9.0–13.5% of body weight). Pyörälä (6) reported that the mean half-life of warfarin in random-bred male Sprague–Dawley rats (18 hr) was shorter than in female rats (28 hr), and he observed that the mean estimated volume of distribution in male rats (13.2% of body weight) was statistically significantly larger than in female rats (11.4%).

The total clearance of warfarin in rats, as determined in this investigation, ranged from 2.60 to 43.0 ml kg^{-1} hr^{-1} (Table II) and may be compared to a much narrower range of 1.39–4.62 ml kg^{-1} hr^{-1} in six normal human adults (16). Because of this narrower range in humans, it is not surprising that available data from a total of only eight normal human subjects (10, 17) do not

reveal any significant relationship between the warfarin elimination rate constant and the plasma warfarin concentration required to elicit a certain degree of inhibition of R_{syn}. Such correlation is more likely to be evident in a group of human subjects consisting of individuals with widely differing warfarin half-lives not ascribable to exposure to enzyme inducers or inhibitors.

The results of this investigation demonstrate that unusually rapid or slow elimination of warfarin may be (at least in some cases) the result of, or associated with, unusually extensive or limited distribution of the drug in extravascular spaces. This, in turn, may affect markedly the relationship between warfarin concentration in the plasma and its anticoagulant effect, since this drug acts in the liver and not (like heparin) in the circulation. It is, therefore, unwise to adjust warfarin dosage regimens of patients solely on the basis of their warfarin half-life without considering the possibility that an unusually long or short warfarin half-life is associated with a change in the relationship between plasma warfarin concentration and intensity of anticoagulant effect. Subsequent reports will deal with the mechanism of the observed intersubject differences in the distribution of warfarin in the body.

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Anticonvulsant Activity and Inhibition of Respiration in Rat Brain Homogenates by Substituted Oxadiazoles

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Abstract □ Several 2,5-disubstituted 1,3,4-oxadiazoles were synthesized, characterized, and tested for their effectiveness in inhibiting the respiratory activity of rat brain homogenate. All substituted oxadiazoles and their precursors, thiosemicarbazides, were found to inhibit nicotinamide adenine dinucleotide- (NAD) dependent oxidations of pyruvate and α-ketoglutarate as well as the NAD-independent oxidation of succinate. Anticonvulsant activity, as exhibited by protection against pentylenetetrazol-induced seizures, with substituted thiosemicarbazides and the corresponding cyclized oxadiazoles ranged from 30 to 90% at a dose of 100 mg/kg. The degree of protection afforded by these compounds, however, was unrelated to their ability to inhibit oxidation of pyruvate, α-ketoglutarate, and succinate.

Keyphrases □ 1,3,4-Oxadiazoles, 2,5-disubstituted—synthesis, inhibition of NAD-dependent and NAD-independent oxidations, relationship to anticonvulsant activity □ Structure-activity relationships—2,5-disubstituted 1,3,4-oxadiazoles-anticonvulsant activity, rats □ Thiosemicarbazides, substituted—synthesis, anticonvulsant activity, inhibition of respiratory activity, rats □ Pyruvate, α-ketoglutarate, and succinate oxidation, inhibition—effects of substituted thiosemicarbazides and cyclized oxadiazoles □ Anticonvulsant activity—relationship to inhibition of respiratory activity, substituted thiosemicarbazides and oxadiazoles

Considerable interest has recently been focused on 2,5-disubstituted 1,3,4-oxadiazoles, which have been shown to possess analgesic (1, 2), central nervous system depressant (3), muscle relaxant (4–6), and tranquilizing (4, 5) properties. These observations prompted the synthesis of 2-arylamino-5-(4-*tert*-butyl-2-bromophenoxymethyl)-1,3,4-oxadiazoles. In the present study, the ability of these oxadiazoles and their precursors, thiosemicarbazides, to inhibit nicotinamide adenine dinucleotide- (NAD) depen-