Some Anticoagulant Properties of 2-Acyl-1,3-indandiones and Warfarin in Rabbits

S. RICHARD HEISEY, J. PALMER SAUNDERS, and KENNETH C. OLSON

Pharmacology Branch, Chemical Corps Medical Laboratories, Army Chemical Center, Md.

The probable mechanism of action of warfarin, 2-pivalyl-1,3-indandione, and other acylindandiones is inhibition of the formation of prothrombin or other coagulation factors in the liver with a concomitant increase in capillary permeability. Warfarin and the acylindandiones profoundly increase prothrombin time in animals receiving small daily doses, but little information has been available concerning their action after single doses. Measurements were made of the ability of warfarin and a series of acylindandiones to increase plasma coagulation time in rabbits after a single dose. Diphenylacetylindandione was the most powerful anticoagulant. Warfarin was relatively ineffective. Some of the less active indandiones and some compounds not included in the single-dose experiments were tested at low diurnal and semidiurnal doses. Isobutyrylindandione was the most active compound in this group. Vitamin K_1 was effective in reversing the effects of diphenylacetylindandione on plasma coagulation time.

ANTICOAGULANT COMPOUNDS have come into widespread use in recent years to combat harmful rodents. This trend was started after Campbell and Link isolated and crystallized the agent responsible for hemorrhagic sweet clover disease (*1*). Link and coworkers identified the compound as 3,3′-methylenebis (4-hydroxycoumarin) and succeeded in synthesizing it (*15*). Its effect on the plasma prothrombin time of various animals was studied (*9*), and later the anticoagulant action of 105 related compounds was compared with that of the 3,3′-methylenebis (4-hydroxycoumarin) (*8*). Compound 42 in this series [3-(α-acetonylbenzyl)-4-hydroxycoumarin], later named warfarin, was studied and found to possess species specificity for susceptibility to its action. The rat was especially affected, which suggested to Link and associates that warfarin might be useful in the control of rodents (*13*).

Warfarin has been used effectively by military and civilian agencies for controlling rodents (*3*, *16*). In use, warfarin is incorporated into cereal baits in a concentration of 0.025%. The rodent feeds on the bait for days, ingesting small daily doses of the rodenticide which eventually result in a hemorrhagic death.

In 1944 Kabat (*6*) described the anticoagulant properties of a series of 2-acyl-1,3-indandiones which previously had been reported by Kilgore (*7*) to possess insecticidal properties. The compound reported to be most effective in both anticoagulant and insecticidal action was 2-pivalyl-1,3-indandione (Pival). Since then Pival has come into general use as a rodenticide similar in activity to warfarin, but possessing the additional advantage of insecticidal action.

The mechanism of action of the 2-acyl-1,3-indandiones seems to be similar to that of the dicoumarol-like compounds in impairing the capacity of the liver to form one or more coagulation factors (*5*, *13*), and in producing capillary damage (*6*, *10*), a combination of actions which may result in a hemorrhagic death.

A crude but convenient method of measuring the anticoagulant action of a compound is the one-stage prothrombin method of Quick (*11*). Although this method does not differentiate among the various factors involved in blood coagulation, it does yield valuable information about the over-all effects of anticoagulant compounds. The work reported here was undertaken to compare various indandiones and related compounds with warfarin for ability to produce an increased plasma coagulation time after oral or parenteral administration.

Procedure

Rabbits of mixed breed and sex were used in these experiments. Plasma coagulation time was measured by a procedure similar to that described by Quick (*11*). Blood (2.5 cc.) was drawn from a marginal ear vein into a syringe containing 0.25 cc. of 0.1*M* sodium oxalate. The sample was then centrifuged at 2000 r.p.m. for 7 minutes, after which the plasma was drawn off. Then 0.1 cc. of oxalated plasma was pipetted into a small test tube (10 × 75 mm.), and 0.1 cc. of thromboplastin was added. After the plasma and thromboplastin had been mixed, 0.1 cc. of 0.02*M* calcium chloride was blown into the mixture from a pipet and a stopwatch was started. The mixture was then agitated and observed while the tube was tilted so that the fluid ran up and down one side of the tube. When the first threads of fibrin clot were observed, the watch was stopped and the time noted. This time was the plasma coagulation time. At least two such determinations were made on each plasma sample. The thromboplastin in the above mixture was made up from stock ampoules of desiccated rabbit brain (Difco). In later experiments 0.2 cc. of a commercial preparation containing thromboplastin and calcium chloride (Simplastin) was added to 0.1 cc. of plasma, and the plasma coagulation time was measured as above. The two procedures gave different normal values of plasma coagulation time. In general, the values obtained by the Simplastin procedure were lower than those obtained using the thromboplastin (Difco) method.

The copper and iron derivatives of the indandiones, 2-benzoyl-1,3-indandione, 2(3-furylacrylyl)-1,3-indandione, 2-isobutyryl-1,3-indandione, 2-heptanoyl-1,3-indandione, 2-propionyl-1,3-indandione, 2-phenylacetyl-1,3-(5-nitro)indandione, isovaleryl phthalimide, benzoyl ethyl acetoacetate, and dibenzoyl pivalylmethane were prepared in these laboratories. The other compounds listed in Tables I and III were obtained from commercial sources.

A typical procedure for testing a compound when administered parenterally is as follows. A normal blood sample was taken from a rabbit, and the plasma coagulation time was determined. The drug was then administered intraperi-

toneally, and coagulation time was determined at 2, 8, 24, 48, 72, and 96 hours thereafter. All the compounds were tested at a single dose of 40 mg. per kg. Those compounds which markedly increased the plasma coagulation time were tested similarly at a dose level of 5 mg. per kg.

Some of the compounds which showed little or no activity at the level of 40 mg. per kg. were tested for their ability to increase plasma coagulation time when administered orally in daily or twice daily doses of 5 mg. per kg. Some compounds not previously tested were also screened in this manner.

To test the therapeutic effectiveness of vitamin K_1, only diphenylacetylindandione was used. This compound was selected because of its known high potency in producing an increase in plasma coagulation time. Rabbits previously injected with 10 mg. per kg. of diphenylacetylindandione intraperitoneally were given 5 mg. per kg. of vitamin K_1 (Mephyton) in saline intravenously at various times after the injection of the anticoagulant. Plasma coagulation time was measured at various time intervals after the injection of the indandione.

Results

Table I shows typical plasma coagulation times obtained after single injections of 40 mg. per kg. of various substances. The compounds tested include warfarin, nine indandiones, and also four compounds of related structures.

Figure 1 is a graphic representation of data derived from the values given in Table I. The highest measured ratio of postinjection plasma coagulation time to preinjection coagulation time is given for each compound tested. The time at which this highest ratio occurred is given in parentheses above each bar. The drugs giving the greatest responses were diphenylacetylindandione (the free acid and the iron salt), pivalylindandione, and phenylacetylindandione.

Table II gives typical plasma coagulation times after single injections of 5 mg. per kg. of those compounds which showed significant anticoagulant activity at the 40 mg. per kg. level.

Figure 2 is a graphic representation of

Table I. Plasma Coagulation Times in Rabbits after Single Intraperitoneal Dose of 40 Mg. per Kg.

Compound	Time after Administration, Hours: 0	2	8	24	48	72	96	No. of[a] Animals
	Experimental Plasma Coagulation Times, Seconds							
Diphenylacetyl-[b]	11.2	11.6	18.3	40.0	150.3	Dead	...	3
Diphenylacetyl- (Fe^{++} cmpd.)[b]	11.7	9.2	12.1	20.1	55.0	>200	>200	1
Pivalyl-[b]	11.2	10.6	11.5	15.6	43.9	>200	>200	2
Phenylacetyl-[b]	8.7	...	14.1	28.2	>200	>200	>200	3
Isovaleryl- (Fe^{++} cmpd.)[b]	8.4	10.3	8.8	19.0	>200	58.0	Dead	2
Acetyl-[b]	5.5	11.7	15.4	25.9	60.1	26.4	32.4	3
Isovaleryl-[b]	9.2	13.1	14.9	32.2	61.4	36.0	13.7	3
Isovaleryl- (Cu^{++} cmpd.)[b]	9.7	13.9	13.0	13.4	43.2	57.5	57.5	3
Warfarin	9.6	12.0	10.2	16.6	36.7	18.3	14.8	2
Benzoyl-[b]	8.0	10.2	11.9	13.8	10.1	9.0	10.3	1
Isovaleryl phthalimide	10.1	9.5	9.0	9.4	11.1	12.9	10.2	2
Benzoyl ethyl acetoacetate	10.1	11.4	10.3	9.2	10.3	12.6	12.2	2
Diacetylisovalerylmethane	9.2	8.7	10.6	12.7	8.3	12.6	16.6	1
Dibenzoylpivalylmethane	9.4	8.6	8.8	9.0	10.2	9.7	9.7	1

[a] Where more than one animal was used, data represent values from single typical experiment.
[b] Indandione.

Table II. Plasma Coagulation Times in Rabbits after Single Intraperitoneal Dose of 5 Mg. per Kg.

Compound	Time after Administration, Hours: 0	2	8	24	48	72	96	No. of[a] Animals
	Experimental Plasma Coagulation Times, Seconds							
Diphenylacetyl-[b]	8.9	10.4	11.0	19.6	47.7	42.7	>200	2
Pivalyl-[b]	9.2	13.2	13.3	20.1	111.5	101.1	67.5	2
Phenylacetyl-[b]	11.5	16.4	11.8	22.1	21.8	60.9	97.1	3
Isovaleryl- (Fe^{++} cmpd.)[b]	9.6	10.1	10.4	12.3	11.5	12.1	10.1	1
Acetyl-[b]	11.0	11.6	12.7	11.8	14.2	15.2	11.7	1
Isovaleryl-[b]	12.1	13.3	12.3	13.2	11.6	10.6	10.8	1
Isovaleryl- (Cu^{++} cmpd.)[b]	10.5	12.7	10.5	11.5	13.7	15.1	10.9	1
Warfarin	7.9	11.6	11.0	21.9	35.2	13.6	11.7	2

[a] Where more than one animal was used, data represent values from single typical experiment.
[b] Indandione.

Table III. Change of Plasma Coagulation Times in Rabbits Given Daily Doses (Per Os) of 1,3-Indandiones[a]

2-R-1,3-indandione, R=	Daily Dose, Mg./Kg.	Time after Administration, Hours: 0	48	72	96
		Experimental Plasma Coagulation Time, Seconds			
Isobutyryl-	5	7.5	12.5	24.0	>200[b]
Propionyl-	5	6.4	9.0	13.5	34.9
Isovaleryl-	5	5.8	7.5	13.2	24.7
Heptanoyl-	5	6.3	14.7	18.7	20.8
Acetyl-	5	5.9	8.0	10.2	13.3
Benzoyl-	5	7.1	6.9	6.4	7.6
3-Furylacrylyl-	5	8.0	6.7	6.8	7.6
Phenyl-	5	7.6	7.8	6.6	6.8

[a] Data from single experiments.
[b] Died in 8 days.

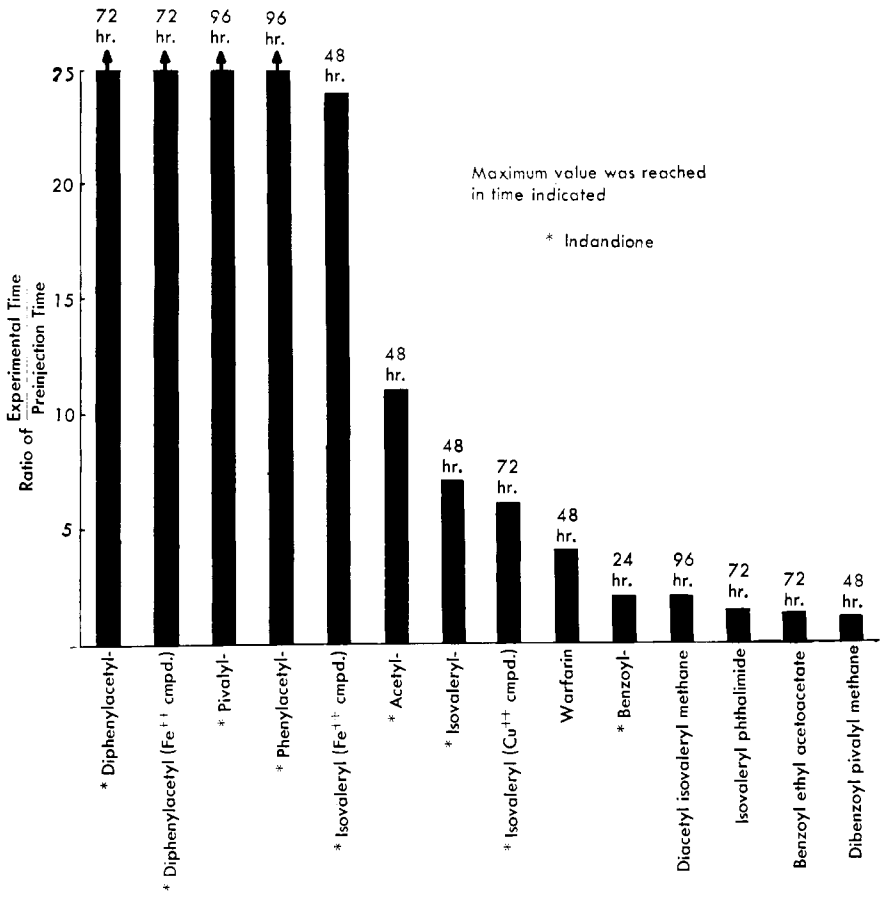

Figure 1. Anticoagulant effect of various compounds in rabbits

Single dose, 40 mg. per kg.

data derived from the values given in Table II, presented in the same manner as in Figure 1. Diphenylacetylindandione is the most effective compound at 5 mg. per kg., followed by pivalyl- and phenylacetylindandione. Although none of these latter compounds could be differentiated at the 40 mg. per kg. level, the lower dose elicited a sharp decline in activity for pivalyl- and phenylacetylindandione. Warfarin appears to be no more effective at 40 mg. per kg. than at 5 mg. per kg.

Table III gives plasma coagulation times of rabbits which were given daily oral doses of indandiones. The compounds are listed in order of decreasing effectiveness. 2-Isobutyryl-1,3-indandione was effective, producing a plasma coagulation time greater than 200 seconds at 96 hours after the initial dose. This animal died 8 days after the first dose. 2-Benzoyl-1,3-indandione, 2-(3-furylacrylyl)-1,3-indandione, and 2-phenyl-1,3-indandione were ineffective in producing a prolonged plasma coagulation time.

Table IV gives plasma coagulation times of rabbits which were given oral doses of indandiones twice daily. Here again, the compounds are ranked in order of decreasing effect. Isovaleryl-indandione was no more effective when given twice daily than when given only once daily. 2-Phenyl-1,3-indandione showed more activity when given twice daily than when given only once daily.

Vitamin K_1 given intravenously appeared to be effective in reversing the anticoagulant action of diphenylacetylindandione. As seen in Table V, if the vitamin K_1 was given 8 or 24 hours after administration of the anticoagulant, the plasma coagulation time never increased beyond normal limits. If the vitamin K_1 was given 48, 72, or 96 hours after the anticoagulant, the rise in plasma coagulation time which had occurred (rising to more than 100 seconds in the 96-hour instance) was restored to near normal levels within 24 hours.

Discussion

Of the compounds considered here, three stand out as very effective in prolonging plasma coagulation time after a single injection. In order of decreasing activity they are: 2-diphenylacetyl-1,3-indandione, 2-pivalyl-1,3-indandione, and 2-phenylacetyl-1,3-indandione. At the dose level of 40 mg. per kg. the toxic action of diphenylacetylindandione was so severe that many of the animals died within 72 hours after injection of the drug. At necropsy the skin was usually blanched and the peritoneal cavity contained large amounts of sanguinous fluid.

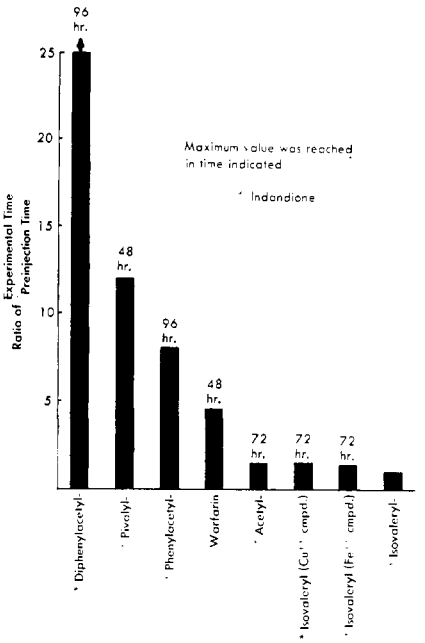

Figure 2. Anticoagulant effect of various compounds in rabbits

Single dose, 5 mg. per kg.

Table IV. Change of Plasma Coagulation Times in Rabbits Given Semidiurnal Doses (Per Os) of 1,3-Indandiones[a]

2-R-1,3-indandione, R=	Semidiurnal Dose, Mg./Kg.	Time after Administration, Hours: 0	48	72	96
		Experimental Plasma Coagulation Time, Seconds			
Acetyl-	5	5.8	13.4	..[b]	..
Isovaleryl-	5	6.0	11.1	12.8	22.9
Phenyl-	5	6.2	11.2	11.7	7.7
Benzoyl-	5	6.2	9.6	8.9	11.1
3-Furylacrylyl-	5	6.2	6.9	6.6	9.2
Phenylacetyl[c]	5	5.9	6.2	6.6	5.6

[a] Data from single experiments.
[b] Animal died.
[c] -1,3-(5-nitro) indandione.

Table V. Effect of Intravenous Vitamin K_1 on Plasma Coagulation Time

(Given at various intervals after intraperitoneal administration of 10 mg. per kg. of diphenylacetylindandione in rabbits)

Time of Intravenous Administration of Vitamin K_1 (5 Mg./Kg.), Hours after DPAI[a]	No.[b] of Animals	Time after Administration, Hours						
		0	24	48	72	96	120	144
		Plasma Coagulation Time, Seconds						
8	4	7.4	9.1	10.1	12.9	7.3	..	..
24	1	6.4	..	..	7.7	6.7	7.0	5.8
48	1	7.8	..	..	7.9	6.9	8.1	6.7
72	2	7.1	..	..	36.7	9.1	9.0	10.3
96	5	7.0	..	..	50.4	106.1	8.4	7.2

[a] Diphenylacetylindandione.
[b] Where more than one animal was used, data represent values from single typical experiment.

If the compound being tested showed any appreciable activity, an increase in the plasma coagulation time was apparent within 24 hours. In many cases the coagulation time was at its highest value at the time of the 96-hour sample, which was the last sample taken in these experiments.

The anticoagulant activity of warfarin seems to have a different pattern than that of the indandiones. When given in a single dose, warfarin was not strikingly effective in producing a prolonged plasma coagulation time, as compared with the indandiones. Tables I and II show that the amount of warfarin injected appeared to have little effect on the extent of increase in plasma coagulation time, which was approximately four times the normal value whether a dose of 5 mg. per kg. or of 40 mg. per kg. was given. This finding is consonant with the low toxicity which warfarin exhibits when given in a single dose to rats (*10,12*).

Although the toxicity of warfarin increases greatly when the compound is given in a series of small daily doses (*12*), Shapiro (*14*) has reported successful clinical use of this drug for the induction of therapeutic hypoprothrombinemia by giving relatively small doses initially (50 to 100 mg.), followed by maintenance doses of 12.5 mg. per day or 25 mg. every other day.

As could be predicted from toxicity studies on the indandiones (*12*), some of the compounds which showed little or no activity after administration of a single dose produced increases in plasma coagulation time after repeated daily or semidiurnal doses (Tables III and IV). Although some of these increases are small when compared to those produced by diphenylacetylindandione, the less active compounds may have significance in the treatment of cardiovascular disease. When anticoagulant drugs are used clinically for the production of hypoprothrombinemia, only small increases in coagulation time are required. 2 - Phenyl - 1,3 - indandione and benzoylindandione showed only slight activity even when administered twice daily, but Fisher and others (*4*) have reported the successful use of phenyl indandione clinically and point out the rapid return of clotting time to normal when administration of the drug is stopped. It is conceivable that benzoylindandione also may be of potential value for clinical use.

Vitamin K_1 (Mephyton-Merck) was found to be effective in reversing the anticoagulant effect of 2-diphenylacetyl-1,3-indandione. In some experiments, the plasma coagulation time exceeded 200 seconds 96 hours after injection of the diphenylacetylindandione. A dose of 5 mg. per kg. of vitamin K_1 returned the coagulation time to pre-injection levels within 24 hours. These findings are in agreement with those of Correll and others (*2*), who found that vitamin K_1 administered orally or intravenously in divided doses of from 20 to 400 mg. per rabbit could reverse the anticoagulant action of diphenylacetylindandione within 24 hours. Correll used a level of anticoagulant activity expressed as 10% of normal "prothrombin," which may mean a plasma coagulation time of 20 to 25 seconds. In the work reported here, even though plasma coagulation time sometimes exceeded 200 seconds, normal values were obtained within 24 hours after a single intravenous injection of 5 mg. per kg. of vitamin K_1.

Acknowledgment

The authors wish to express their appreciation to the following for their generosity in supplying the experimental compounds: Motomco, Inc., for 2-pivalyl-, 2-isovaleryl-, 2-acetyl-, and 2-phenylacetyl-1,3-indandiones; Upjohn Co. for 2-diphenylacetyl-1,3-indandione (Dipaxin); Shell Development Co. for diacetylisovalerylmethane; and Merck & Co., Inc., for vitamin K_1 (Mephyton). They also wish to express appreciation to Alfred D. Goldstone and Ernest C. Bay for their technical assistance.

Literature Cited

(1) Campbell, H. A., and Link, K. P., *J. Biol. Chem.* **138,** 21–33 (1941).
(2) Correll, J. T., Coleman, L. L., Long, S., and Willy, R. F., *Proc. Soc. Exptl. Biol. Med.* **80,** 139–43 (1952).
(3) Favorite, F. G., *Med. Technicians' Bull. (Armed Forces Med. J.)* **3,** 89–96 (1952).
(4) Fisher, M. M., Wilensky, N. D., Griffith, R. W., Drumm, A., Diefenbach, A. E., and Frankel, G. J., *N. Y. State J. Med.* **54,** 778–83 (1954).
(5) Hunter, R. B., and Shepherd, D. M., *Brit. Med. Bull.* **11,** 56–61 (1955).
(6) Kabat, H., Stohlman, E. F., and Smith, E. I., *J. Pharmacol. and Exptl. Therap.* **80,** 160–70 (1944).
(7) Kilgore, L. B., Ford, J. H., and Wolfe, W. C., *Ind. Eng. Chem.* **34,** 494 (1942).
(8) Overman, R. S., Stahmann, M. A., Huebner, C. F., Sullivan, W. R., Spero, L., Doherty, D. G., Ikawa, M., Graf, L., Roseman, S., and Link, K. P., *J. Biol. Chem.* **153,** 5–24 (1944).
(9) Overman, R. S., Stahmann, M. A., Sullivan, W. R., Huebner, C. F., Campbell, H. A., and Link, K. P., *Ibid.*, **142,** 941–55 (1942).
(10) Public Health Service Clinical Memorandum, Technical Development Bulletin, 1st Revision, Communicable Disease Center, Savannah, Ga., May 1, 1952.
(11) Quick, A. J., *Am. J. Clin. Pathol.* **10,** 222 (1940).
(12) Saunders, J. P., Heisey, S. R., Goldstone, A. D., and Bay, E. C., J. AGR. FOOD CHEM. **3,** 762–65 (1955).
(13) Seegers, W. H., *Pharmacol. Revs.* **3,** 278–344 (1951).
(14) Shapiro, S., *Angiology* **4,** 380–90 (1953).
(15) Stahmann, M. A., Huebner, C. F., and Link, K. P., *J. Biol. Chem.* **138,** 513–27 (1941).
(16) Yelton, V., *Med. Technicians' Bull. (Armed Forces Med. J.)* **2,** 277–8 (1951).

Received for review September 8, 1955. Accepted November 4, 1955.