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A METHOD FOR MEASURING THE ACTIVITY OF COMPOUNDS WITH AN ACTIVITY LIKE VITAMIN K AGAINST INDIRECT ANTICOAGULANTS IN RATS

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A method for the estimation of the activity of compounds like vitamin K using rats treated with an indirect anticoagulant is described. The assay measured the speed of onset and magnitude of the antidotal effect. Under the conditions employed, only vitamin K_1 produced a significant response. Vitamin K_3 and its water-soluble derivatives were inactive. Both the magnitude and the speed of onset of the antidotal effect of vitamin K_1 were found to depend on the extent to which it was in solution. The assay gave results of fair accuracy and reproducibility, which permitted a full statistical analysis and provided an estimate of error.

The biological assay of compounds with vitamin K activity in the vitamin-deficient chick mainly estimates the dietary activity; it does not give an indication of the ability to reverse the hypoprothrombopaenia caused by indirect anticoagulants of the dicoumarol type. For example, in the chick assay vitamin K_3 (2-methyl-1:4-naphthoquinone) is more active than vitamin K_1 (2-methyl-3-phytyl-1:4-naphthoquinone), whereas as an antidote against anticoagulant substances such as dicoumarol vitamin K_1 is more active and shows a more rapid onset of action. Almquist (1954) has made a detailed review of the factors involved in the biological estimation of vitamin K active compounds.

The assay reported here measures the effect of vitamin K active compounds in the rat made hypoprothrombopaenic by giving an indirect anticoagulant, and therefore gives a more specific estimate of the antidotal activity than the chick assay. Under the conditions of the assay, which allow the determination of the magnitude and speed of onset of antidotal effect, only vitamin K_1 has been found to possess activity, while vitamin K_3 and its water soluble derivatives were found to be inactive.

METHODS

Preparation of Animals.—Male rats weighing from 175 to 225 g. were given an oral dose of 0.5 g. of warfarin (3-[α-acetonylbenzyl]-4-hydroxycoumarin) mixed with 5 g./animal/day of feed (hulled oats) for 2 days. The animals were fed at 4 p.m. and the assay performed 17 to 20 hr. after the second dose, when the prothrombin time (" bedside method ": see below) had increased from a normal value of 30 sec. to over 300 sec. Special feed dishes were used to prevent scattering of feed, and animals which failed to eat all their ration were rejected from the assay. Preliminary experiments showed that the addition of the anticoagulant to the feed gave a more uniform increase of the prothrombin time than intraperitoneal or intravenous injection.

Withdrawal of Blood Samples.—Blood samples were drawn from a lateral tail vein. The rats were restrained either by being wrapped tightly in a towel or by being lightly anaesthetized with ethyl ether. To draw blood samples consistently by tail vein puncture certain technical details had to be carefully observed. The tail was warmed in water at 45 to 50° with gentle massaging for about 1 min. or until the veins were well dilated. Too high a temperature caused collapse of the veins. Blood was drawn with a 27 gauge $\frac{3}{4}$ in. needle and a 0.5 ml. tuberculin syringe. The plunger of the syringe fitted the barrel tightly. If necessary, stopcock grease was used to obtain a close fit. The rate of drawing the blood was adjusted to the rate of flow in order to avoid collapse of the vein. All glassware was scrupulously clean, particularly the needle, since the presence of any trace of material with thromboplastic activity may induce clotting due to the slow rate at which the blood is drawn. Needles were treated with Arquad (Jaques, 1955) and syringes were coated with silicone (Jaques, 1955). Many investigators have been discouraged from using the technique of drawing blood from the tail vein of the rat because of the difficulties encountered before

skill is acquired. Attention to the details listed above may avoid some of these difficulties.

For the "bedside method," blood was drawn without anticoagulant. For the proconvertin method, exactly 0.1 ml. of blood was drawn into 0.4 ml. of a buffered citrate solution (Dyggve and Lund, 1954). The sample was placed in a plastic test tube (75 × 10 mm.) and centrifuged at 2,500 rev./min. for 10 min. The plasma obtained thus will be referred to as diluted citrated plasma.

Prothrombin Time Determination: Bedside Method (A).—This was a modification of the "bedside method" of Schwager and Jaques (1949). One drop of rabbit brain thromboplastin suspension and one drop of freshly drawn whole blood were placed separately on a 1.5 in. watch glass. As the two drops were mixed with a needle, a stopwatch was started and the mixture was stirred gently until a distinct clot formed at the tip of the needle.

Method for the Specific Determination of Proconvertin (B).—This was a modification of the micromethod of Dyggve and Lund (1954) for the specific determination of proconvertin. The test plasma consisted of 0.1 ml. of diluted citrated plasma in a silicone coated test tube (75 × 10 mm.) to which was added 0.1 ml. of sodium citrate solution, 0.2 ml. proconvertin-free bovine plasma and 0.2 ml. rabbit brain thromboplastin. The determination was carried out in a water bath at 37° and was started by adding 0.1 ml. $CaCl_2$ of optimal concentration: this was determined for each batch of proconvertin-free bovine plasma. The proconvertin-free bovine plasma was prepared according to the method of Biggs and MacFarlane (1957).

Vitamin K_1 Preparations.—The vitamin K_1 for the preparation of the standard was obtained from commercial sources and purified by column chromatography (Binkley, MacCorquodale, Thayer, and Doisy, 1939). The vitamin was dissolved in benzene, absorbed on Folin's Permutite and eluted with benzene/petrol ether (20%/80% v/v); small amounts of impurities were retained on the column. The eluate was concentrated *in vacuo* at 30° and the vitamin stored in closed ampoules protected from light.

Vitamin K (40 mg.) was dissolved in Tween 80 (0.5 ml. polyoxyethylene sorbitan mono-oleate, Atlas Powder Co.). The vitamin was mixed carefully with Tween 80 to give a homogeneous paste and water was added in small quantities, the mixture being worked carefully into a homogeneous suspension after each addition and finally made up to 10 ml. The final suspension was completely clear. If the vitamin was not mixed carefully with Tween 80 before the addition of water, or if all the water was added at once, a turbid suspension resulted which possessed considerably less activity. Tween 80 at the concentration used did not change the prothrombin time.

The other two preparations tested were vitamin K_1 suspensions at present marketed under proprietary names.

Results

Preliminary experiments indicated that the vitamin K_1 standard dissolved in Tween 80 could be sensitively assayed between 10 and 80 μg./animal. It produced an optimal antidotal effect after 30 min. Tables I and II show the response to graded doses after 30 min. when the determination of the prothrombin time was carried out by the bedside method (method A) and the proconvertin method (method B) respectively.

In the sensitive assay range, the reciprocal of the prothrombin time was found to be a rectilinear function of the logarithm of the dose, and the data were analysed after this transformation. The results of the standard analysis of variance are shown in Tables I and II. For both methods the mean square due to linear regression was highly significant, and that due to deviation from linear regression was not significant when tested against the residual mean square due to deviation within doses. The assay using method B was carried out on two consecutive days. The mean square due to variations between days was just significant at the 5% level of probability (Table II).

Figs. 1 and 2 show the log dose/response curves fitted by the method of least squares with the exact 95% confidence limits for the estimated most probable log dose.

TABLE I

ANTIDOTAL ACTIVITY OF VITAMIN K. PROTHROMBIN TIME OF RATS PRETREATED WITH WARFARIN (METHOD A)

Prothrombin times (sec.) for each animal.

	Vitamin K_1 (μg./Animal)			
	10	20	40	80
	70	59	48	37
	95	65	38	40
	113	108	63	38
	226	219	86	35
	380	136	44	
	200	102	49	
Mean	187·3	115·0	54·6	37·5

Analysis of Variance

Source of Variance	Sum Sq.	d.f.	Mean Sq.	F	*P*
Regression ..	1,090·4	1	1,090·4	55·7	< 0·001
Deviations from linearity ..	42·4	2	21·2	1·08	> 0·20
Between doses ..	1,132·8	3			
Residual error ..	53·2	18	19·6		
Total ..	1,486·0	21			

b: −22·25±2·94 (s.e.). λ: 0·199.

b is the slope of the regression line and $\lambda = \sqrt{\frac{\text{Mean Square Error}}{b}}$

TABLE II
ANTIDOTAL ACTIVITY OF VITAMIN K_1. PROTHROMBIN TIME OF RATS PRETREATED WITH WARFARIN (METHOD B)

Prothrombin times (sec.) for each animal.

	Vitamin K_1 (μg./Animal)			
	10	20	40	80
1st day ..	360 193 346 295	232 240 203	225 127 123 130	99 112 87
2nd day ..	515 324 483	240 201 112 185	88 119 119	80 113 93 94
Mean ..	359	202	133	97

Analysis of Variance

Sources of Variance	Sum Sq.	d.f.	Mean Sq.	F	*P*
Regression	219·249366	1	219·249366	172	< 0·001
Deviations from linearity	0·225816	2	0·112908	12·195	< 0·10 > 0·05
Between doses ..	219·475182	3			
Between days ..	8·240553	1	8·240	5·95	< 0·05 > 0·01
Residual error ..	31·887376	23	1·386408		
Total ..	259·60311	27			

b: $-8·31 \pm 0·66$ (s.e.). λ : 0·143.

TABLE III
ASSAYS OF ANTIDOTAL ACTIVITY OF VITAMIN K_1 (METHOD B)

Date	2/57	4/57	1/58	1/58	2/58
No. animals ..	15	28	12	15	12
,, dose levels ..	3	4	3	3	3
Slope (b) ± s.e. ..	−17·04 ±4·43	−8·31 ±0·66	−8·56 ±1·46	−11·61 ±2·12	−10·06 ±2·26
λ	0·248	0·143	0·134	0·173	0·184

When the dose is estimated from the curve for method A (Fig. 1) an estimated dose of 10 μg. does not differ significantly from an estimated dose of 20 μg., but the differences between the estimations of the other three dose levels are significant ($P<0.05$). For method B, the differences between all four dose levels are significant ($P<0.05$).

Technically, method B is the more convenient, since plasma samples can be set aside for later determinations. In method A, the prothrombin time determinations have to be carried out immediately, an inconvenience when the prothrombin times tend to be long and many samples have to be taken at specified intervals.

Table III summarizes five assays using method B and shows that the results are reproducible over long periods of time.

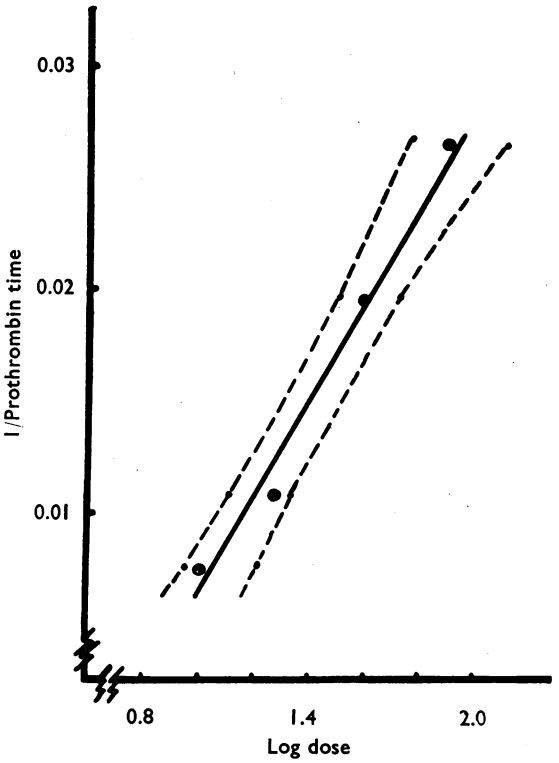

FIG. 1.—Log dose/response line and 95% confidence limits for vitamin K_1 standard determined by method A. Log dose in μg./animal.

The assay has been used to estimate the antidotal activity of two proprietary colloidal solutions of vitamin K_1. Preliminary experiments showed that preparation 1 produced no effect after 30 min., a slight effect after 1 hr., and an optimal antidotal effect only after 2 hr. Preparation 2 showed some activity after 30 min. and optimal antidotal activity after 1 hr. The potency of the two preparations has been estimated against the standard in 4-point parallel line assays. The responses to 20 and 80 μg./animal of the standard after 30 min. and to 100 and 400 μg./animal of the test preparations are shown in Tables IV and V respectively. The effect of preparation 1 was measured after 2 hr. and that of preparation 2 after 1 hr. The standard analysis of variance for a 4-point parallel line assay showed that, for both assays, the mean squares due to regression were highly significant, whereas those due to deviation from parallelism are not significant (Tables IV and V). The relative potencies of preparations 1 and 2 in terms of the standard are 0.250 and 0.256

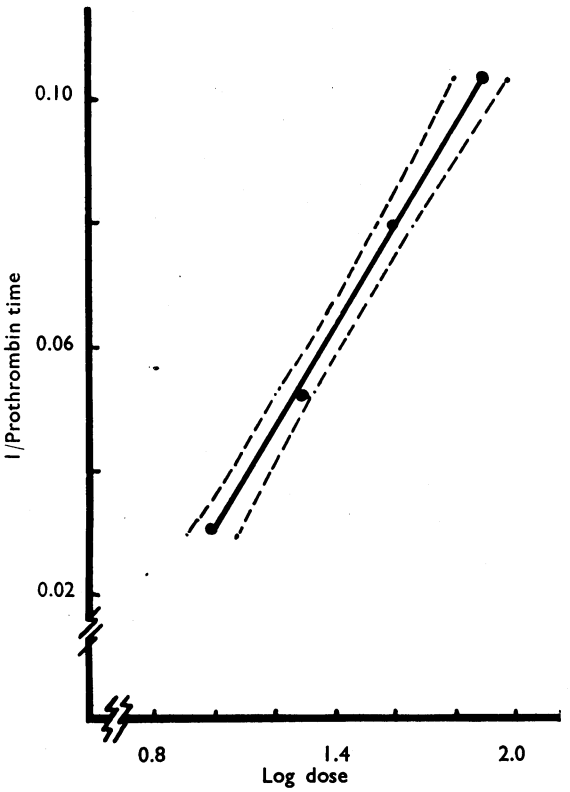

FIG. 2.—Log dose/response line and 95% confidence limits for vitamin K_1 standard determined by method B. Log dose in μg./animal.

respectively; the differences are statistically significant for both preparations.

DISCUSSION

The antidotal activity of vitamin K active compounds against indirect anticoagulant drugs is determined by the magnitude and speed of onset. Of these two factors, the speed of onset is important when vitamin K active compounds are used for the correction of excessively prolonged prothrombin times, for the arrest of haemorrhage due to overdosage of anticoagulant drugs, or for the preparation of a patient under anticoagulant treatment for surgery, when it is desirable to return the prothrombin time to normal as rapidly as possible. Although it has been recognized clinically that the onset of the antidotal effect of vitamin K_1 is more rapid than that of vitamin K_3 and its water-soluble derivatives, the use of the chick assay to estimate the potency of vitamin K active compounds has tended to obscure these differences. The assay described here measures

TABLE IV

COMPARISON OF ANTIDOTAL ACTIVITIES OF VITAMIN K_1 PREPARATION 1 AND VITAMIN K_1 STANDARD (METHOD B)

Five animals/treatment. Prothrombin times in sec.

Doses in μg./animal:	Standard		Preparation 1	
	20	80	100	400
	232	99	168	92
	240	112	117	64
	203	87	232	97
	240	80	167	87
	201	113	169	66
Mean	223	98	171	81

Analysis of Variance

Source of Variance	Sum Sq.	d.f.	Mean Sq.	F	P
Preparations ..	19·523	1	10·523	6·901	< 0·05
Regression ..	192·572	1	192·572	68·070	< 0·001
Parallelism ..	9·585	1	0·585	4·948	> 0·20
Between doses ..	212·680	3			
Residual error ..	45·259	16	2·829		
Total ..	257·939	19			

b: −20·62. λ: 0·081. Log rel. potency (M) ± s.e. = −0·603 ± 0·109. Rel. potency (R) = 0·250. 95% limits of R: 0·189 − 0·337.

TABLE V

COMPARISON OF ANTIDOTAL ACTIVITIES OF VITAMIN K_1 PREPARATION 2 AND VITAMIN K_1 STANDARD (METHOD B)

Six animals/treatment. Prothrombin times in sec.

Doses in μg./animal:	Standard		Preparation 2	
	20	80	100	400
	189·0	48·0	90·9	47·5
	122·0	44·0	84·3	56·4
	157·8	51·0	112·6	26·3
	106·0	39·5	91·9	46·3
	116·0	47·6	111·7	40·1
	92·2	65·8	129·1	52·6
Mean	160·5	49·3	103·4	45·9

Analysis of Variance

Sources of Variance	Sum Sq.	d.f.	Mean Sq.	F	P
Preparations ..	32·900	1	32·900	1·795	> 0·10
Regression ..	1,051·521	1	1,051·521	57·391	< 0·001
Parallelism ..	1·995	1	1·995	9·183	> 0·20
Between doses	1,086·416	3			
Residual error	366·431	20	18·322		
Total ..	1,452·847	23			

b: −21·99. λ: 0·195. Log rel. potency (M) ± s.e. = −0·592 ± 0·078. Rel. potency (R) = 0·256. 95% limits of R: 0·214–0·306.

the antidotal activity under experimental conditions which approximate more closely to those encountered clinically and therefore gives a more direct estimate of the relative potency. Although the assay has been designed to estimate antidotal activity against anticoagulant drugs

such as coumarin, it could also be used to estimate vitamin K activity in biological material in place of the chick assay. It is likely to be found more convenient in laboratories which lack facilities for work with the chick or want to avoid the preparation of vitamin K deficient diets.

Previous attempts to use the rat for the bioassay of vitamin K active compounds have not been entirely satisfactory because of the difficulties encountered in making the animal deficient by dietary means (Greaves, 1939). Special procedures (such as ligation of the bile duct) which interfere with the absorption of the vitamin from the intestine have been tried (Flynn and Warner, 1940). Quick and Stefanini (1948) showed that the chick pretreated with dicoumarol can be used for the estimation of vitamin K.

Under the conditions of the assay, which involved a significant reduction of the prothrombin time within 2 hr., vitamin K_3 and its water-soluble derivatives failed to show any activity even when given in such large doses that they produced toxic effects. This observation differs somewhat from the results of Dam and Søndergaard (1953), who observed that, in the vitamin K deficient chick, vitamin K_3 caused a decrease of the elevated prothrombin time during this interval. In a vitamin K deficient animal, the primary defect is in the synthesis or availability of the vitamin. If vitamin K_3 served as a precursor for a more complex derivative, the rate of synthesis of this derivative in the vitamin deficient animal might be sufficient to correct the coagulation defect within 2 hr. In contrast, the mode of action of the indirect anticoagulant drugs is generally believed to be due to competitive inhibition with vitamin K. To overcome this inhibition, larger amounts of the active derivative, in excess of the rate of synthesis, may be necessary. The more complex vitamin K_1, on the other hand, may act directly or with only slight structural changes. The difference in activity of the three vitamin K_1 preparations tested appears to be due to the extent to which the fat-soluble, water-insoluble vitamins were in solution. Dam and Søndergaard (1953) also used Tween 80 for dissolving vitamin K_1 and showed that this preparation had a very rapid onset of action in the vitamin K deficient chick. Although the three preparations tested differed significantly in activity, the parallelism of the dose/response curves indicated that their mode of action was nevertheless the same.

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