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The Rabbit as an Animal Model for Warfarin-Induced Hypoprothrombinemia: The Relationship between Prothrombin Time and Prothrombin Complex Activity

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The rabbit model of warfarin-induced hypoprothrombinemia was statistically investigated in terms of the relation between prothrombin time (PT) and prothrombin complex activity (PCA). Among various thromboplastin reagents examined, Simplastin-A was used because of its relatively distinct endpoint in the manual tilt-tube method and convenient coagulation time. The PCA 60%, selected as a measure for assessing the coagulation recovery induced by various oral vitamin K_1 preparations after experimental hypoprothrombinemia in the previous work (Nagata *et al.*, *J. Pharm. Pharmacol.*, **36**, 527 (1984)), was confirmed to be a reasonable criterion to judge whether the coagulation recovery reaches normal based on a regression analysis of the compiled standard curves of PCA ($n=194$). The regression equation was given by $\log PT(s) = 1.78 - 0.428 \times \log PCA$ (%), $r=0.996$. The criterion of PCA 20% or less for maximal warfarin-induced hypoprothrombinemia was also justified ($13.5 \pm 8.5\%$, $n=102$). The advantages of the rabbit model include repeated availability of the same animal (at intervals of more than 3 weeks), and the convenient PT difference between PCA 60% and 20% (6—8 s).

Keywords——animal model; rabbit model; warfarin-induced hypoprothrombinemia; prothrombin time; prothrombin complex activity; thromboplastin reagent

The rabbit has been used as an *in vivo* model for evaluating the thrombosis-inducing activity of coagulation factors[1-3] and the effects of anticoagulants.[4-6] For the purpose of evaluating the biological efficiency of vitamin K_1 preparations, we have attempted to establish an animal model of warfarin-induced hypoprothrombinemia using rabbits. We have used the model rabbits for the evaluation of various oral vitamin K_1 preparations including liposome-associated vitamin K_1.

The induction of hypoprothrombinemia was characterized by a prothrombin complex activity (PCA) of 20% or less and the efficiency of blood coagulation recovery was estimated in terms of the time required for the PCA to return to 60% after vitamin administration.[7] The PCA 60% was adopted simply following the criterion for coagulation recovery used by Yacobi *et al.*,[8] though no evidence was given for its validity.

The present communication describes the choice of an appropriate thromboplastin reagent for the rabbit blood coagulation test. The criterion of PCA 60% was rationalized on the basis of a statistical analysis of the relationship between the prothrombin time (PT) and the PCA covering 194 examples. The criterion of PCA 20% or less established for warfarin-induced hypoprothrombinemia (12 mg/kg *i.v.*) is also discussed on the basis of the reference PCA data ($n=102$).

Experimental

Animals and Materials——Male Japanese white rabbits weighing 3.0—4.0 kg were maintained in a temperature-controlled room (25 °C) with access to water (500 ml/d) and normal pelleted feed (200 g/d), and were fasted for one

night before use. Thromboplastin reagents were commercially obtained: Thrombotest Owren (lot no. 652, Eisai Co., Ltd., Tokyo), Thromboplastin · C (lot no. TPCD-184 A, DADE, Miami, Florida), and Simplastin-A (20 different lots including lot no. 1691099-4027092, Warner-Lambert, Morris Plains, New Jersey). Ci-TROL (lot no. COLl-160 and COLl-174, DADE, Miami, Florida), and prothrombin-free rabbit plasma (lot no. RP-64, DADE) were commercially obtained. They were reconstituted according to the manufacturers' instructions. Warfarin (racemate) was prepared by dissolving its potassium salt (Eisai Co., Ltd., Tokyo) and used as an equimolar sodium salt. All other chemicals used were of reagent grade.

PT and the Standard Curve of PCA——Thirty-five rabbits were randomly used. Each rabbit was used 5.5 times (4 to 7 times) on average, after recovery to normal from induced hypoprothrombinemia in each case. PT was determined by Quick's one-stage method.[9] Blood samples (1.8 ml) were collected from the marginal ear vein into 3.8% trisodium citrate (0.2 ml) using a disposable injector and centrifuged at 3000 rpm for 10 min at room temperature (25°C). A 0.1 ml aliquot of citrated plasma was diluted with 0.9% NaCl (0.1 ml), and half of the mixture was mixed with 0.2 ml of the thromboplastin reagent. Solutions were preincubated at 37°C for 1—3 min before mixing. The time required for clot formation was measured by the manual tilt-tube method. An average clotting time of five determinations was taken for each sample; the mean deviation was within 1.5% about the mean value in every case. To check whether reproducible and precise values of PT are obtained, the coagulation control reagent (Ci-TROL) was used as a normal control on a routine basis. To normalize PT, a standard curve of PCA in every experiment on each rabbit was constructed based on a series of geometric dilutions (100—3.2%) with 0.9% NaCl.[10] Because the activity of coagulation factors in rabbit plasma was higher than that in human plasma, the 50% dilution was assumed to be PCA 100% (Fig. 1) and 50%-diluted plasma is hereinafter referred to as normal plasma.

Warfarin-Induced Hypoprothrombinemia——Before production of the experimental hypoprothrombinemia, each rabbit was examined to establish the standard curve of PCA mentioned above. Rabbits were then treated with warfarin 12 mg/kg *i.v.* This dose was selected for the induction of hypoprothrombinemia based on the effect of warfarin dose on the time required for the PCA to return to 60%.[7] The rabbits were fasted for 22—24 h after being dosed with the anticoagulant and then the PT was measured. The hypoprothrombinemia was characterized by a PCA value of 20% or less and was generally induced in each animal at intervals of at least three weeks to avoid possible complications due to enzyme induction or enzyme-related changes.[11]

Statistical Analysis——The relationship between PCA treatment groups and PT was analyzed by the one-way classification.[12] Regression analysis is based on the assumption that the distribution of the PT values corresponding to a predetermined PCA value is normal and that the relationship of log PT *vs.* log PCA is linear. The reference values of normal (represented by PT) and hypoprothrombinemia (represented by PCA) were determined based on the assumption that 95% of all observations would fall within two standard deviations from the mean.[13]

Results and Discussion

Selection of Thromboplastin Reagent

To select an appropriate thromboplastin reagent for the rabbit blood coagulation test from commercially available reagents, which are primarily prepared for the human blood coagulation test, the values of PT in various 0.9% NaCl dilutions of normal plasma were investigated for rabbit plasma with reference to human plasma (Ci-TROL reconstituted) for Thrombotest Owren, Thromboplastin · C and Simplastin-A, as shown in Fig. 1.

Rabbit plasma generally showed 20—40% shorter PTs than human plasma with all reagents, indicating that coagulation factors of rabbits are more active. Among the reagents examined, Simplastin-A showed a practically linear dependency of the PT on the plasma dilution ratio ranging from 50 to 3.2%, giving PTs of about 10 to 30 s, respectively, but the response deviated from linearity for intact rabbit plasma. Thrombotest Owren required longer coagulation times which may be inconvenient in measuring low coagulation activities. Thromboplastin · C showed unclear endpoints when plasma was diluted less than 25%. However, when prothrombin-free rabbit plasma containing factors I and V was added, this reagent was almost equivalent to Symplastin-A. Accordingly, Simplastin-A was employed because of its relatively distinct end-point in the manual tilt-tube method and convenient coagulation time.

PT of Normal Rabbits

The paired *t*-test was used for comparison of normal PTs for untreated rabbits with those for rabbits treated with warfarin:[14] $p<0.05$ was obtained for each pair regardless of the

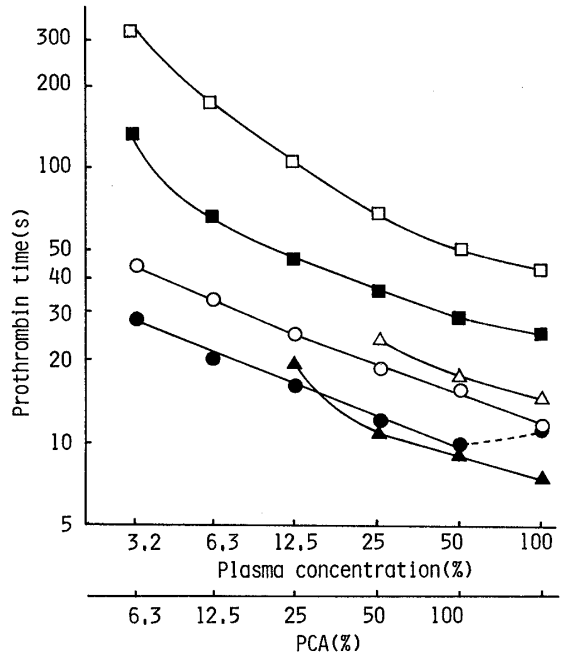

Fig. 1. The Relationship between the Dilution Ratio (or PCA) and the PT for Human (Ci-TROL Reconstituted) and Rabbit Plasma

Open symbols, human; closed symbols, rabbit. Thromboplastin reagents: Thrombotest Owren (□ and ■); Simplastin-A (○ and ●); Thromboplastin·C (△ and ▲). For human plasma each point is the mean of 4—5 determinations. For rabbit plasma each point is the mean of 2—4 animals.

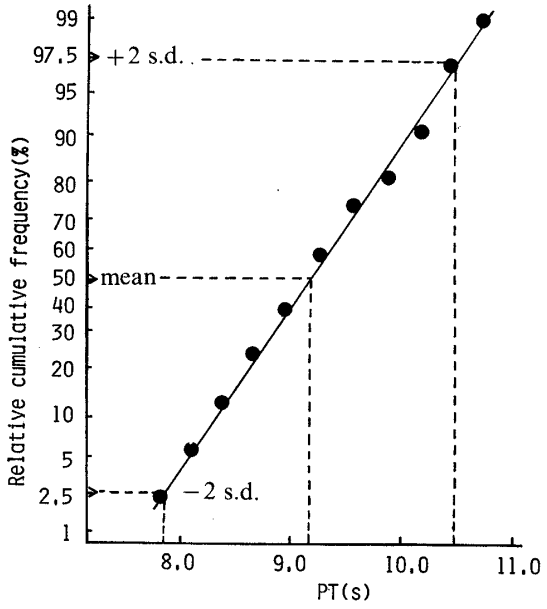

Fig. 2. Cumulative Frequency Distribution of Normal PT Plotted on Normal Probability Paper

The total number of PT observed was 194, and PT ranged from 7.7 to 11.2 s. Class interval, 0.3 s.

number of treatments. The number of PTs compiled for normal plasma totalled 194. The frequency distribution for the PTs (ranging from 7.7 to 11.2 s) based on 14 classes with a class interval of 0.3 s was symmetrical. The cumulative frequency distribution was plotted on normal probability paper, as shown in Fig. 2. The reference PT was assumed to take values ranging from 2.5 to 97.5% (two standard deviations from the mean) on the ordinate, where a satisfactorily linear relation was obtained.[13] The reference PT value of normal plasma with 95% confidence limits was 9.2 ± 1.3 s.

Regression Analysis of Standard Curves of PCA

A standard curve of PCA was constructed for each sample by diluting normal plasma with 0.9% NaCl and measuring the PT values. The data consist of five groups of PCA, each of which contains 194 observations of PT. The hypothesis that the relation between PT and PCA can be described by a linear equation describing a geometric curve was verified by analysis of variance (95% confidence).[14] The regression equation of the relationship was:

$$\log \mathrm{PT(s)} = 1.78 - 0.428 \times \log \mathrm{PCA(\%)} \qquad (1)$$

$$(r = 0.996,\ n = 970)$$

The 95% confidence intervals of the PT regression line were constructed on the basis of the estimated regression line as follows;

$$t(968,\ 0.05)\sqrt{0.002\left(\frac{1}{970} + \frac{(\mathrm{PCA(\%)} - 38.8)^2}{70.2}\right)}$$

Accordingly, when PCA = 100%, the PT with 95% confidence intervals was 8.6 ± 0.6 s. The

TABLE I. Simulated PT Values at PCA 60% and 20% for Different PT Values of Normal Plasma

PT of normal (s) PCA 100%	PT (s)		$b^{a)}$
	PCA 60%	PCA 20%	
10.5	13.2	20.9	1.88
9.2	11.5	18.6	1.82
7.9	9.8	15.5	1.75

a) $\log PT = b - 0.428 \times \log PCA$.

confidence intervals of Eq. 1 may also be applicable to judging whether a standard curve of PCA constructed for an untreated rabbit or a rabbit that might have undergone repeated inductions of hypoprothrombinemia deviates from normal.

The actual region of the reference PT values estimated earlier was broader than that constructed on the basis of the regression line for the standard curves of PCA. This is because the former is based on the assumption that 95% of all observations would fall within two standard deviations from the mean, the region of which is regarded as the reference value of untreated rabbits. The latter indicates the precision with which the conditional mean is estimated and does not imply the extent of distribution of the individual values of observed PT.

Assuming that the prolongation tendency of PT with plasma dilution expressed by Eq. 1 is applicable to dilutions of individual normal plasmas having individual values of PT, we could calculate a PT corresponding to a given PCA, provided that the first term of the right-hand side of Eq. 1 is adjusted since the PT of normal plasma is always assumed to be equivalent to the PCA 100% for each sample. For the upper limit (10.5 s), the lower limit (7.9 s) and the mean (9.2 s) within the normal PT range assigned to PCA 100%, the simulated PT values at PCA 60% and 20% are shown in Table I. Because of the geometric relation between PT and PCA, the precision of the conversion of PT to its corresponding PCA value abruptly declines above about PCA 60%. Thus, the difference of PTs between PCA 100% and 60% was much less than that between 60% and 20%.

When the coagulation recovery is checked during recovery from hypoprothrombinemia to normal, a criterion to judge whether the coagulation time has returned to normal must be established, because we need to know the time required for PCA to return to normal.[7] However, PTs obtained experimentally show considerable variance of the corresponding PCA values as the coagulation recovery approaches normal, *i.e.* in the region giving PCA values more than about 60%. The PCA 60%, therefore, is considered to be a reasonable upper limit as a criterion for evaluation of the coagulation recovery.

PCA in Warfarin-Induced Hypoprothrombinemia

To induce experimental hypoprothrombinemia in rabbits, we investigated the effect on the PT of warfarin at a dose of 12 mg/kg, chosen to give the maximal inhibition of clotting time.[7] The number of the PT values compiled was 102. The longest and shortest PTs were 29.5 and 13.5 s, respectively, and the range was 16.0 s. When the raw data were plotted with a class interval of 1.6 s, the frequency distribution appeared to have positive skew and the mean (18.7 s) did not coincide with the median (21.5 s) or the mode (18.0 s).

The PT values in hypoprothrombinemia appeared to be much more scattered than those of the normal rabbits. These PT values, however, are raw data that have not been normalized to their normal PTs. Therefore, to eliminate the influence of rabbit-to-rabbit variance and lot-to-lot variance of the thromboplastin reagent, each PT value was converted to the

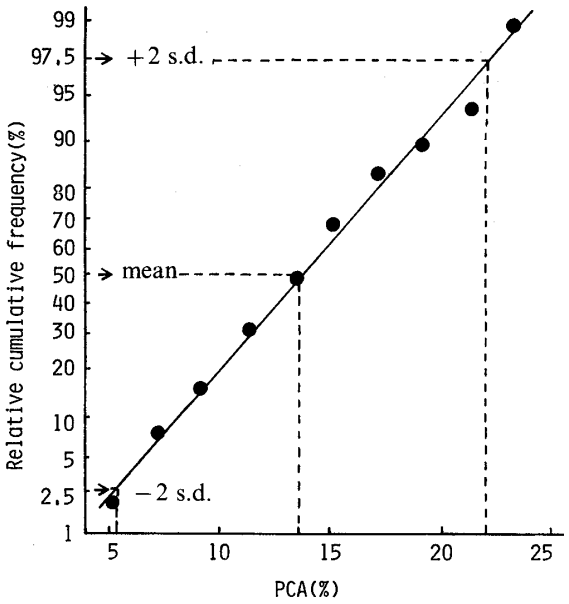

Fig. 3. Cumulative Frequency Distribution of PCA in Hypoprothrombinemic Rabbits Plotted on Normal Probability Paper

The total number of PCA obtained was 102, and PCA ranged from 5.5 to 25.5%. Class interval, 2.0%.

corresponding PCA according to an individual standard curve of PCA constructed for every experiment on each rabbit.

The frequency distribution was expressed in terms of the PCA with 13 classes for the data ranging from PCA 5.5 to 25.5%, with a class interval of 2%. The mean was symmetrical (13.5%), and almost coincided with the median (15.5%) and the mode (15.5%). The cumulative frequency distribution was plotted on normal probability paper, as shown on Fig. 3. The PCA of hypoprothrombinemic rabbits was likewise estimated from the linear portion with 95% confidence limits and was $13.5 \pm 8.5\%$.

The number of the compiled data is far more than that in the previous report.[7] Accordingly, it is reasonable to consider that warfarin-induced hypoprothrombinemia in the rabbits can be characterized by a PCA value of 20% or less, because the upper limit of the PCA obtained statistically is 22%.

The advantages of the use of rabbits include the fact that relatively frequent blood samplings are possible from the same animal for each run, and repeated uses of the same animal are possible at intervals of more than 3 weeks without causing any significant changes of blood coagulation characteristics. The convenient PT difference between PCA 60% and 20% is also an advantage. The results should be useful in developing an animal model for warfarin-induced hypoprothrombinemia using rabbits.

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