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A Route Dependent Bioavailability in Active Metabolite Suggested in Riboflavin-5′-Phosphate Pharmacokinetics

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The pharmacokinetics of riboflavin-5′-phosphate (FMN) and the metabolite riboflavin (FR) in the rat was studied with measurement of the plasma levels after the injection into femoral and portal vein of the FMN dose of 1 and 10 mol. Area under the curve (AUC) of FR plasma level *vs.* time after FMN injection into portal vein was found to be larger than the case of femoral vein. This route dependency of AUC in active metabolite was successfully explained from viewpoint of the compartment theory of linear pharmacokinetics. The AUC of FR was also varied by the dose of FMN. This dose dependency was analyzed to be the decrease in total clearance of FR with increasing FMN dose. The model dependent rate constants in FMN and FR pharmacokinetics did not vary with the dose and route but the distribution volume of FR did. The per cent of FR excreted in bile and urine in 24 hours after injection of FMN did not vary with the dose and route of FMN injection.

Keywords——riboflavin-5′-phosphate; riboflavin; prodrug; pharmacokinetics; linear compartment theory; route dependency; area under the curve; deconvolution calculation

Area under the curve (AUC), which represents the time course of blood or plasma level of a drug, is called AUC and admitted widely to be an index of the bioavailability.[2] The AUC normalized by the ingested dose is well known to vary sometimes with the ingestion route, the absorption velocity, and even the dose. The route dependency has been elucidated successfully by the compartment theory[3] as well as the perfusion theory.[4] The dose dependency has also been expressed by the non-linear pharmacokinetics.[5] In spite of the successful explanation, it has been limited to AUC of drug ingested by itself, but the elucidation for AUC of metabolite after ingestion of precursor or pro-drug has not yet been made. The present report dealt with the pharmacokinetics of riboflavin-5′-phosphate (FMN) and the metabolite riboflavin (FR) in the rat, and discussed the route and dose dependency of the AUC from the viewpoint of the compartment theory.

Route Dependent Bioavailability of a Metabolite after Ingestion of Precursor

The route dependency of AUC has been expressed in relationship between both rate constants of the drug transport from liver to central circulation system and of the drug elimination in liver according to the compartment theory. Fig. 1 illustrates the simplest compartment system to express the route dependency of AUC of a metabolite after the ingestion of precursor. The addition of peripheral compartments in which precursor is not cleared has

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3) *a*) M. Gibaldi and S. Feldman, *J. Pharm. Sci.*, **58**, 1477 (1969); *b*) M. Gibaldi, R.N. Boyes, and S. Feldman, *J. Pharm. Sci.*, **60**, 1338 (1971).
4) M. Rowland, L.Z. Benet, and G.G. Graham, *J. Pharmacokin. Biopharm.*, **1**, 123 (1973).
5) K.S. Albert, E. Sakmar, M.R. Hallmark, D.J. Weidler, and J.G. Wagner, *Clin. Pharmacol. Ther.*, **16**, 727 (1974).

been proved to preserve essential relationship concerning the route dependency, and conversion of the compartment model to the perfusion theory has also been reported.[6]

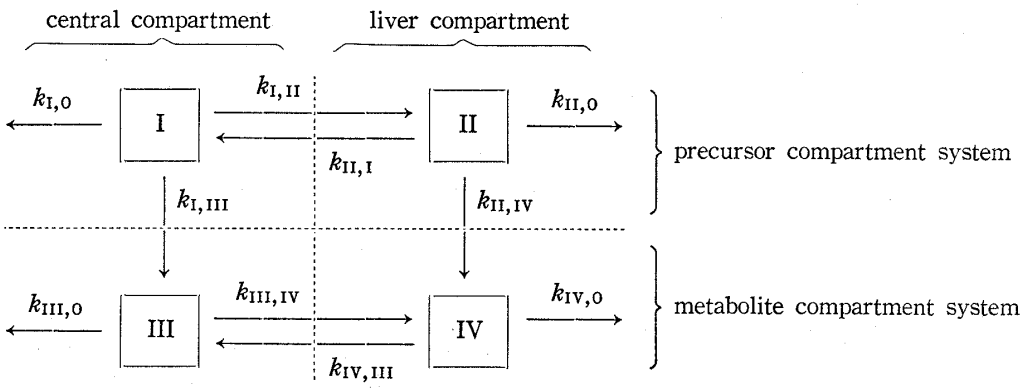

Fig. 1. Compartment Model illustrating Ingestion Route Dependent Bioavailability in Precursor-Metabolite System
$k_{i,j}$: the first order rate constant from compartment i to j.

According to treatment described by Segre, *et al.*[7] for the liner compartment system, the next simultaneous equations of matrix formula express the model shown in Fig. 1.

$$\begin{pmatrix} s+K_I & -k_{II,I} & 0 & 0 \\ -k_{I,II} & s+K_{II} & 0 & 0 \\ -k_{I,III} & 0 & s+K_{III} & -k_{IV,III} \\ 0 & -k_{II,IV} & -k_{III,IV} & s+K_{IV} \end{pmatrix} \begin{pmatrix} x_I \\ x_{II} \\ x_{III} \\ x_{IV} \end{pmatrix} = \begin{pmatrix} f_I \\ f_{II} \\ 0 \\ 0 \end{pmatrix} \tag{1}$$

In equation 1, x_i (i=I, II, III, IV) is the Laplace transform of amount in compartment i, s is independent variable in the transform, k is the rate constant illustrated in Fig. 1, f_I and f_{II} are the input functions, *i.e.* the Laplace transform of the absorption velocity into the respective compartments of I and II, which are numerically equal with the dose in the case of bolus injection into the corresponding compartment, and K_i is the sum of outflow rate constants from the compartment i and is expressed as follows.

$$\begin{aligned} K_I &= k_{I,0} + k_{I,II} + k_{I,III} \\ K_{II} &= k_{II,0} + k_{II,I} + k_{II,IV} \\ K_{III} &= k_{III,0} + k_{III,IV} \\ K_{IV} &= k_{IV,0} + k_{IV,III} \end{aligned} \tag{2}$$

The definition of AUC gives the way of direct calculation from the Laplace transform of amount in the compartment as follows,

$$\mathrm{AUC} = \int_0^\infty \frac{X}{V} dt = \frac{1}{V} \lim_{s \to 0} \int_0^\infty \exp(-s \cdot t)\, X\, dt = \frac{1}{V}\, x\, (s=0) \tag{3}$$

where V represents the distribution volume of the compartment.

Thus, ratio of AUC (R_1) in precursor after the portal vein injection by itself to that after the femoral vein injection is calculated by ratio between two values of x_I which are solved from equation 1 in which null and unit are substituted for f_I and f_{II} in the numerator, respectively, and the just counter substitutions are used in the denominator. In both cases, the variable s in the solved x_I is limited to null.

6) S. Awazu, T. Oguma, T. Iga, and M. Hanano, *Chem. Pharm. Bull.* (Tokyo), **25**, 680 (1977)
7) A. Rescigno and G. Segre, "Drug and Tracer Kinetics," Blaisdell Publishing Co., 1966.

$$R_1 = -\frac{\begin{vmatrix} -k_{II,I} & 0 & 0 \\ 0 & K_{III} & -k_{IV,III} \\ -k_{II,IV} & -k_{III,IV} & K_{IV} \end{vmatrix}}{\begin{vmatrix} K_{II} & 0 & 0 \\ 0 & K_{III} & -k_{IV,III} \\ -k_{II,IV} & -k_{III,IV} & K_{IV} \end{vmatrix}} = \frac{k_{II,I}}{K_{II}} = \frac{1}{1+\dfrac{k_{II,o}+k_{II,IV}}{k_{II,I}}} \quad (4)$$

In the same manner, ratio of AUC (R_2) in the metabolite is calculated by x_{III} solved from equation 1 as follows.

$$R_2 = -\frac{\begin{vmatrix} K_I & -k_{II,I} & 0 \\ -k_{I,III} & 0 & -k_{IV,III} \\ 0 & -k_{II,IV} & K_{IV} \end{vmatrix}}{\begin{vmatrix} -k_{I,II} & K_{II} & 0 \\ -k_{I,III} & 0 & -k_{IV,III} \\ 0 & -k_{II,IV} & K_{IV} \end{vmatrix}} = \frac{K_I k_{II,IV} k_{IV,III} + k_{II,I} k_{I,III} K_{IV}}{k_{I,II} k_{II,IV} k_{IV,III} + K_{II} k_{I,III} K_{IV}} \quad (5)$$

Equation 4 shows that R_1 is less than or equal to unit. Namely, extent of AUC when drug is intaked through portal vein or absorbed from entero-intestinal track is not more than that when the same drug is injected intravenously in the same dose, as well known. On the other hand, R_2 expressed in equation 5 can be larger than unit as easily shown if $k_{I,III}$ is limited to null as follows.

$$R_2(k_{I,III}=0) = \frac{K_I}{k_{I,II}} = 1 + \frac{k_{I,o}}{k_{I,II}} \quad (6)$$

Extent of AUC in metabolite when precursor is absorbed from entero-intestinal track can exceed that when the precursor is injected intravenously in the same dose. Namely, the oral ingestion can have larger bioavailability than the intravenous injection in the case of pro-drug administration. When $k_{I,III}$ is not limited to null, R_2 can be expressed as follows.

$$R_2 = 1 + \frac{(k_{I,o}k_{II,IV}k_{IV,III} - k_{II,o}k_{I,III}K_{IV} - k_{I,III}k_{II,IV}k_{IV,o})}{(k_{I,II}k_{II,IV}k_{IV,III} + K_{II}k_{I,III}K_{IV})} \quad (7)$$

As shown in equation 7, existence of $k_{I,III}$ concerning the metabolism of precursor in the central compartment decreases R_2. Then, increases of $k_{II,o}$ and $k_{IV,o}$ that concern the elimination of precursor and metabolite in the liver compartment, respectively, as well as decrease of $k_{I,o}$ concerning the elimination of precursor in the central compartment also decrease R_2. Of course, R_2 can be less than unit by these rate constants.

Compartment Analysis of FMN and FR Pharmacokinetics

FR has been studied on the absorption, the metabolism, and the excretion in urine and bile.[8] The quick conversion to FR from FMN in body as well as the time courses of plasma level of FMN and FR in body was also reported.[9] The pharmacokinetics, however, has not yet been analyzed. The compartment model shown in Fig. 2 is assumed for this kinetic study from the following observation. Both time courses of plasma level of FMN and FR in the rat after intravenous bolus injection by themselves are well expressed as the two exponential curve. The metabolic rate process from FMN to FR is presumed to be irreversible. The recovery of FR in urine and bile after FMN injection is not complete.

Organ and tissue that belong to the central or peripheral compartment for FMN are assumed to also belong to the same compartment for even FR in the model shown in Fig. 2. But addition of the rate constants of k_{14} and k_{23} that represent the metabolic rate constant in organ or tissue belonging to the different compartment between FMN and FR does not

8) S. Christensen, *Acta pharmacol. et toxicol.*, **32**, 7, supple II (1973).
9) S. Christensen, *Acta pharmacol. et toxicol.*, **27**, 41-48 (1969).

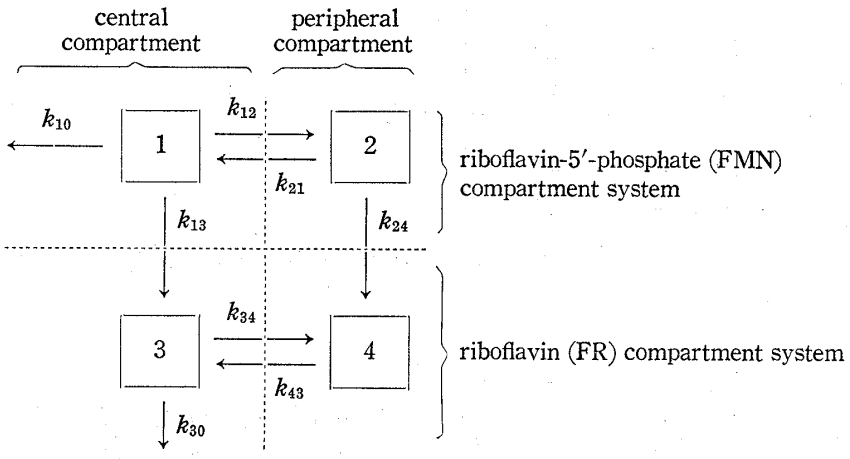

Fig. 2. Compartment Model for Riboflavin-5′-phosphate and Riboflavin Pharmacokinetics

affect the conclusion described later in this report except the value of rate constant dependent on the model.

Since the metabolic rate constant of FMN in the peripheral compartment, k_{24}, can be negligible as described later, the central compartment 1 shown in Fig. 2 is presumed to include both central compartment I and liver compartment II shown in Fig. 1. For the sake of this inclusion of compartment under the assumption of rapid equilibrium, there exist the following relationship between both models.

$$X_1 = X_I + X_{II} = X_I\left(1 + \frac{k_{I,II}}{k_{II,I}}\right) \tag{8}$$

$$k_{13}X_1 = k_{I,III}X_I + k_{II,IV}X_{II} \tag{9}$$

In these equations, X_i represents the amount in the compartment i. From equation 8 and 9, k_{13} is expressed as follows.

$$k_{13} = \left(\frac{k_{I,II}}{k_{I,II}+k_{II,I}}\right)k_{II,IV} + \left(\frac{k_{II,I}}{k_{I,II}+k_{II,I}}\right)k_{I,III} \tag{10}$$

In the same manner, k_{10} is also expressed as follows.

$$k_{10} = \left(\frac{k_{I,II}}{k_{I,II}+k_{II,I}}\right)k_{II,O} + \left(\frac{k_{II,I}}{k_{I,II}+k_{II,I}}\right)k_{I,O} \tag{11}$$

The simultaneous equations of matrix formula for the model shown in Fig. 2 is expressed as follows,

$$\begin{pmatrix} s+K_1 & -k_{21} & 0 & 0 \\ -k_{12} & s+K_2 & 0 & 0 \\ -k_{13} & 0 & s+K_3 & -k_{43} \\ 0 & -k_{24} & -k_{34} & s+K_4 \end{pmatrix} \begin{pmatrix} x_1 \\ x_2 \\ x_3 \\ x_4 \end{pmatrix} = \begin{pmatrix} f_1 \\ 0 \\ 0 \\ 0 \end{pmatrix} \tag{12}$$

where the notation is as same as in equation 1 except name of the compartment, and K's are expressed as follows.

$$\begin{aligned} K_1 &= k_{10} + k_{12} + k_{13} \\ K_2 &= k_{21} + k_{24} \\ K_3 &= k_{30} + k_{34} \\ K_4 &= k_{43} \end{aligned} \tag{13}$$

The Laplace transforms of the plasma levels of FMN and FR after the intravenous bolus injection by FMN, *i.e.* c_1 and c_3, are obtained from the solution of equation 12 for x_1 and x_3, respectively, and expressed as follows,

$$c_1 = \frac{A_1}{s+\alpha_1} + \frac{A_2}{s+\alpha_2} \tag{14}$$

$$c_3 = \frac{B_1}{s+\alpha_1} + \frac{B_2}{s+\alpha_2} + \frac{B_3}{s+\alpha_3} + \frac{B_4}{s+\alpha_4} \tag{15}$$

where $-\alpha_1$ and $-\alpha_2$ are roots of the next quadratic equation,

$$(s+K_1)(s+K_2) - k_{12}k_{21} = 0 \tag{16}$$

and $-\alpha_3$ and $-\alpha_4$ are the roots of the next, similarly.

$$(s+K_3)(s+K_4) - k_{34}k_{43} = 0 \tag{17}$$

The coefficients in equation 14, A's, are expressed as follows where the dose of FMN and the distribution volume of the compartment 1 are shown by D and V_1, respectively.

$$\begin{aligned} A_1 &= \frac{D}{V_1} \cdot \frac{K_2-\alpha_1}{\alpha_2-\alpha_1} \\ A_2 &= \frac{D}{V_1} \cdot \frac{K_2-\alpha_2}{\alpha_1-\alpha_2} \end{aligned} \tag{18}$$

Similarly, B's in equation 16 are expressed as follows,

$$\begin{aligned} B_1 &= \frac{D}{V_3} \cdot \frac{(K_2-\alpha_1)(K_4-\alpha_1)k_{13}-k_{12}k_{24}k_{43}}{(\alpha_2-\alpha_1)(\alpha_3-\alpha_1)(\alpha_4-\alpha_1)} \\ B_2 &= \frac{D}{V_3} \cdot \frac{(K_2-\alpha_2)(K_4-\alpha_2)k_{13}-k_{12}k_{24}k_{43}}{(\alpha_1-\alpha_2)(\alpha_3-\alpha_2)(\alpha_4-\alpha_2)} \\ B_3 &= \frac{D}{V_3} \cdot \frac{(K_2-\alpha_3)(K_4-\alpha_3)k_{13}-k_{12}k_{24}k_{43}}{(\alpha_1-\alpha_3)(\alpha_2-\alpha_3)(\alpha_4-\alpha_3)} \\ B_4 &= \frac{D}{V_3} \cdot \frac{(K_2-\alpha_4)(K_4-\alpha_4)k_{13}-k_{12}k_{24}k_{43}}{(\alpha_1-\alpha_4)(\alpha_2-\alpha_4)(\alpha_3-\alpha_4)} \end{aligned} \tag{19}$$

where V_3 is the distribution volume of the compartment 3.

Equation 14 indicates that the parameters, α_1, α_2, A_1 and A_2, can be evaluated by means of the curve fit of the FMN plasma level after the intravenous injection by FMN itself in the two exponential formula. Similarly, the parameters, α_3, α_4, B_1, B_2, B_3, and B_4, can be expected to be evaluated from the curve fit of the FR plasma level after the injection by FMN from equation 15. This curve fit is difficult due to too many parameters to be detected. Application of the deconvolution calculation, however, can overcome this difficulty. The transfer function from c_1 to c_3, *i.e.* the ratio function of c_3 to c_1, is expressed from the solution of equation 12 as follows.

$$\begin{aligned} \frac{c_3}{c_1} &= \frac{V_1}{V_3} \cdot \frac{\begin{vmatrix} -k_{12} & s+K_2 & 0 \\ -k_{13} & 0 & -k_{43} \\ 0 & -k_{24} & s+K_4 \end{vmatrix}}{\begin{vmatrix} s+K_2 & 0 & 0 \\ 0 & s+K_3 & -k_{43} \\ -k_{24} & -k_{34} & s+K_4 \end{vmatrix}} \\ &= \frac{V_1}{V_3} \cdot \frac{\{k_{12}k_{24}k_{43}+k_{13}(s+K_2)(s+K_4)\}}{(s+K_2)(s+\alpha_3)(s+\alpha_4)} \end{aligned} \tag{20}$$

From rearrangement of this equation, the next is obtained,

$$\frac{c_3}{c_1} = \frac{D_2}{s+K_2} + \frac{D_3}{s+\alpha_3} + \frac{D_4}{s+\alpha_4} \tag{21}$$

where D_2, D_3, and D_4 are expressed as follows.

$$D_2 = \frac{V_1}{V_3} \cdot \frac{k_{12}k_{24}k_{43}}{(\alpha_3-K_2)(\alpha_4-K_2)}$$
$$D_3 = \frac{V_1}{V_3} \cdot \frac{k_{12}k_{24}k_{43}+(K_2-\alpha_3)(K_4-\alpha_3)k_{13}}{(K_2-\alpha_3)(\alpha_4-\alpha_3)} \quad (22)$$
$$D_4 = \frac{V_1}{V_3} \cdot \frac{k_{12}k_{24}k_{43}+(K_2-\alpha_4)(K_4-\alpha_4)k_{13}}{(K_2-\alpha_4)(\alpha_3-\alpha_4)}$$

As shown in equation 22, the value of D_2 becomes null when k_{24} is negligible, *i.e.* the negligible rate of the metabolism from FMN to FR in the peripheral compartment. The weight function which is the reverse Laplace transform of the transfer function, *i.e.* equation 21, is therefore expressed by the two exponential formula when k_{24} is null, but by the three exponential when it can not be negligible. Moreover, K_2 which is the rate parameter of exponential term accompanied by D_2 is calculated by the following equation.

$$K_2 = \frac{A_1\alpha_2+A_2\alpha_1}{A_1+A_2} \quad (23)$$

The contribution of k_{24} to this kinetics can be estimated from the curve fit of the weight function and the comparison between the rate parameters estimated and K_2 calculated. As described later, the weight function of this experiment is always expressed by the two exponential formula, both α_3 and α_4 are proved to be different from K_2, and thus the negligible k_{24} becomes evident.

Experimental

Drug Administration and Sampling——Male albino rats (Donryu) weighing 280—310 g were used. The cannulations to bile duct, urinary bladder, and femoral artery were operated for the samplings of bile, urine, and blood, respectively.[10] Ten or one μmol per head of FMN or nearly saturable dose (about 0.6 μmol) per head of FR was administered by the bolus injection into femoral or portal vein in each experiment. Blood samples were taken 2, 5, 10, 15, 20, 30, 45, 60, and 120 minutes after the injection and both bile and urine samples were taken 0.25, 0.5, 1, 2, 4, 6, 8, and 24 hours after the injection. Light ether anesthesia was used for the operation.

Analytical Methods——FMN and FR in the samples were determined by the fluorometric assay reported by Burch, *et al.*[11] with minor modification as follows. Blood sample (0.3 ml) was centrifuged for 10 min at 3000 rpm at 0—5° in order to separate plasma. To 100 μl of plasma obtained, 5 ml of 5% CCl_3COOH aqueous solution was added to remove protein in ice water in dark. After kept for 5 min, it was centrifuged for 10 min at 12000 rpm. To 3 ml of supernatant, 0.5 ml of 3.67 M K_2HPO_4 aqueous solution was added to neutralize it in a light-resistant test tube. After a sufficient shaking, 1 ml of the neutralized supernatant was diluted by water to 3 ml and this dilute is called the test solution A. To another 2 ml of the neutralized supernatant, 4 ml of benzylalcohol saturated with water was added and shaken vigorously for 15 min in dark for the removal of FR. After centrifugation at 0—5° for 10 min at 3000 rpm, 1.5 ml of the lower layer was washed with 2 ml of $CHCl_3$ saturated with water to remove the remaining benzylalcohol. After standing for 10 min, 1 ml of the upper layer was diluted by water to 3 ml and this dilute is called the test solution B. The test solutions A and B were determined the relative fluorescence intensity at 522 nm excited at 377 nm with Hitachi MPF-4 Fluorospectrometer using 0.25 μg/ml quinine sulfate in 0.1 N sulfuric acid as the standard.

For bile and urine, 0.4 ml of the sample was taken and 5 ml of 5% CCl_3COOH aqueous solution was added to remove protein in ice water in dark. The other procedures were similar to that described above, but 11.74 μg/ml puinine sulfate in 0.1 N sulfuric acid was used as the standard in these cases.

Amount of FR and FMN in a sample were obtained from the following simultaneous equation with the fluorescence intensities.

$$X = A_1x + A_2y$$
$$Y = B_1x + B_2y$$

where X and Y were the relative fluorescence intensities of the test solutions of A and B, x and y were amounts (μ mol) of FMN and FR in the sample, and A_1, A_2, B_1, and B_2 were the coefficients determined from the standard curve.

10) H. Nogami, M. Hanano, S. Awazu, and T. Iga, *Chem. Pharm. Bull.* (Tokyo), **18**, 228 (1970).
11) H.B. Burch, O.A. Bessey, and O.H. Lowry, *J. Biol. Chem.*, **175**, 457 (1948).

Curve Fit and Deconvolution Calculation——For nonlinear curve fit by the least square method and the deconvolution calculation, Hitachi 8700—8800 digital computer was used in the computer center of the university of Tokyo. Nonlinear curve fit was carried out by the use of OS7 HSAP statistical analysis library CMNLR2 of Hitachi Laboratory.[12] This is one of the modified steepest slope method with the weight equal to the reciprocal of the square of observed values. In the convolution and deconvolution calculation, programs developed by ourselves and reported elsewhere[13] were used.

Results and Discussion

AUC of FMN and FR

Fig. 3 and 4 show the time courses of plasma level of FMN and FR after the bolus injections by FMN into femoral and portal vein, respectively. The solid lines in the Fig. 3 and 4

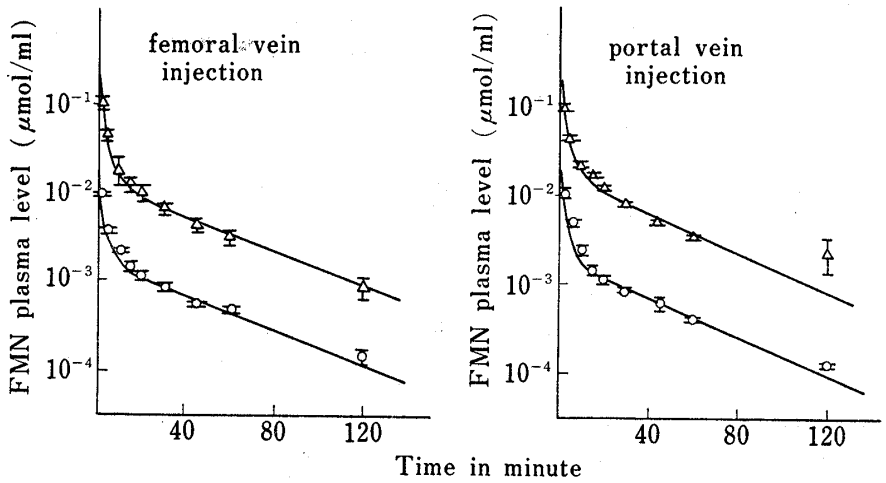

Fig. 3. Time Courses of FMN Plasma Level after Bolus Injection of FMN into Femoral and Portal Vein

Solid lines: Calculated by the two exponential formula with average parameters of three rats. Triangles and circles with column: Averages of observed values in the dose of 10 and 1 μ mol with standard error.

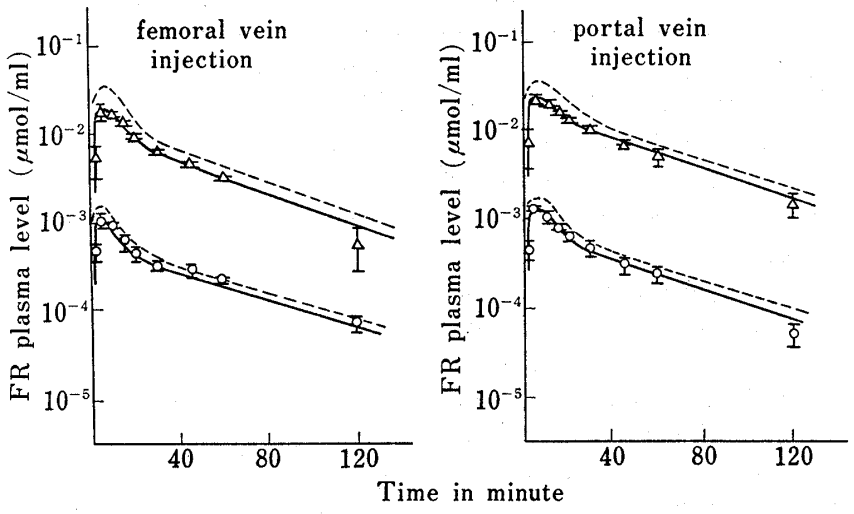

Fig. 4. Time Courses of FR Plasma Level after Bolus Injection of FMN into Femoral and Portal Vein

Solid and dotted lines: Calculated by the convolution of the two exponential formula with average parameters with and without the lag time correction. See in text in details. The other keys: See in Fig. 3.

12) OS7 Hitachi Statistical Analysis Program, 8700-7-004-02 Tokyo, Japan, 1973.
13) H. Kiwada, K. Morita, M. Hayashi, S. Awazu, and M. Hanano, *Chem. Pharm. Bull.* (Tokyo), **25**, (1977),

are drawn from the calculated values by the pharmacokinetic analysis as described later. In Table I, extents of AUC in FMN and FR plasma levels calculated by the parameters obtained from the pharmacokinetic analysis are listed, but every AUC is normarized by the dose of FMN injected, ten and one μmol.

Table I. Area Under the Curve of Plasma Level of FMN and FR *vs.* Time after FMN Injection into Femoral and Portal Vein, Normalized by the Dose

Dose	Route	AUC[a]	
		FMN	FR
1 μ mol	Femoral vein	110.2± 6.9	35.5±2.5
	Portal vein	116.4± 8.3	41.2±4.9
10 μ mol	Femoral vein	96.5±12.0	59.8±2.9
	Portal vein	104.7± 4.3	85.1±3.9

a) calculated by the parameters obtained from the pharmacokinetic analysis. (μM min/μmol) Data are averages of three rats and (±) standard error.

Table II. F-Values in Analysis of Variance of the Normalized AUC of FMN and FR shown in Table I

Factors	Dose	Route	Dose × Route
FMN	1.744	<1	<1
FR	64.958[b]	13.337[a]	1.773

a, *b*) Significance at the levels of 1% and 5%.

The analysis of variance testifying to the dependency of ratio of AUC to the dose on the ingestion route and dose is shown in Table II. As shown in Table I and II, ratio of AUC of FMN to the dose is hardly affected by the ingestion route and dose. In the other words, the first pass effect of liver concerning FMN can be said to be negligible. On the other hand, ratio of AUC of FR to the dose after ingestion of FMN increases always with increase in the dose, and that after portal vein injection is always larger than after the femoral vein injection in each dose of FMN. These differences in dose and route are statistically significant with the levels of 1 and 5%, respectively. The dependency on the dose suggests that pharmacokinetics of FR contains the non-linear process as described later in details. The dependency of AUC of FR on the injection route of FMN is contrary to the usual tendency of the first pass effect. The route dependency found in the present study is consistent in tendency with that proved theoretically as the possibility from the compartment theory. As described before, the FMN elimination out of the central compartment brings this route dependency. Existence of the elimination process of FMN is suggested experimentally as described later in details, but the further evidence is needed in order to confirm the correspondence of experiment with theory. The route dependency resultant by non-linear pharmacokinetics concerning FR plasma level should be denied experimentally. Moreover, the actual elimination rate constant out of liver in FMN and the inflow rate constant to liver that are possibly observed by further experiment should be proved to explain the mechanism expressed by equation 5 without contradiction. The effect of dose on the route dependency can hardly be large, because the interaction factor in analysis of variance is not significant statistically, the route dependency is found in both doses, and the increases in FR plasma level with increase in the dose is very larger than that with difference in the route.

Pharmacokinetics

The time courses of FMN plasma level shown in Fig. 3 is approximated very well by the two exponential formula. The correlation coefficient between the observed and calculated values is 0.9975 with the average value of 12 experiments, and is 0.985 with the minimum. Fig. 5 shows the time course of the weight function calculated by the deconvolution between plasma levels of FR and FMN after injection of FMN.

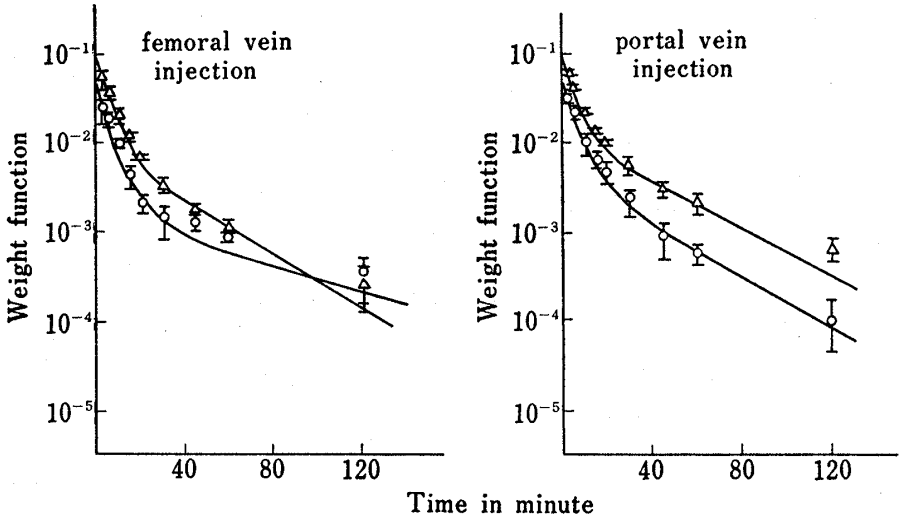

Fig. 5. Time Courses of the Weight Function Calculated by the Deconvolution between Plasma Levels of FR and FMN after Injection of FMN

Solid lines: Calculated by the two exponential formula with average parameters of three experiments. The other keys see in Fig. 3.

From the curve fit of the weight function values to the three exponential formula, the converged covariance matrix can not be obtained by the non-linear least square method which includes the solution of inversion matrix of the normal equation[14] and also the converged parameters can not be obtained by even the steepest slope method. The trial of the curve fit to the three exponential formula under the fixed parameter of K_2 which is calculated by equation 23 is also unsuccessful in order to get the converged parameters. By the two exponential formula however, is very well approximated the time course. The correlation coefficient between the weight function values obtained from the deconvolution and calculated by the two exponential formula is 0.9872 with the average value of 12 experiments, and is 0.895 with the minimum. Besides, both rate coefficients, α_3 and α_4, in the two exponential formula are obviously different from the value of K_2 calculated by the expression for FMN plasma level as described later. The evidence of negligible D_2 in equation 21, *i./e.* the minor contribution of k_{24} in the model shown in Fig. 2, is therefore given by the pharmacokinetic analysis.

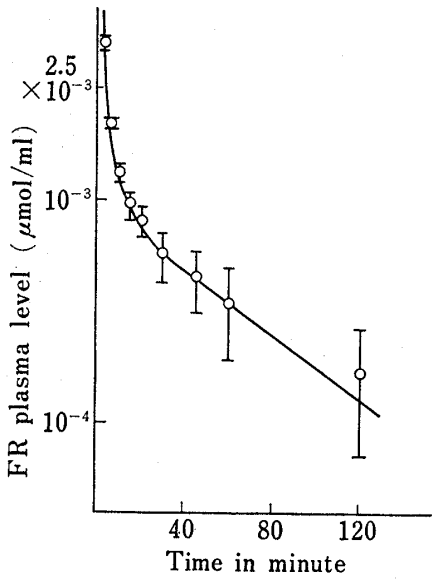

Fig. 6. Time Course of FR Plasma Level after Bolus Injection of FR into Femoral Vein

Solid line: Calculated by the two exponential formula with average parameters of three rats. Circle with column: Averages of observed values in the dose of 0.56 μ mol with standard error.

14) H. Nogami, M. Hanano, S. Awazu, and H.H. Moon, *Chem. Pharm. Bull.* (Tokyo), **17**, 2097 (1969).

Fig. 6 shows the time course of FR plasma level after bolus injection of FR itself with dose of about 0.56 μmol. Because the injected solution is close to the saturation of FR, the injected dose varies slightly in each experiment. This time course is well approximated by the two exponential formula.

Table III lists the values of parameters calculated by curve fit in the two exponential curve of the FMN plasma level, the weight function, and the FR plasma level after the injection by itself. The values of K_2 calculated by equation 23 are also listed in the Table III for the comparison with α_3 and α_4.

Table III. Parameters Calculated by Curve Fit in the Two Exponential Expression of the FMN Plasma Level, the Weight Function, and the FR Plasma Level after the Injection by Itself

Dose	Route	Drug in plasma	Coefficient	Coefficient	K_2	Distribution volume
1 μ mol FMN	Femoral vein	FMN	A_1 0.02274 ±0.00303	α_1 0.5166 ±0.0336	0.07440 ±0.00723	V_1 40.9 ±8.3
			A_2 0.002306 ±0.000086	α_2 0.02873 ±0.00304		V_2 148.8 ±7.6
		Weight function	D_3 0.03388 ±0.01013	α_3 0.1719 ±0.0241	—	V_3 247.9 ±45.6
			D_4 0.002278 ±0.000927	α_4 0.02419 ±0.00502		V_4 400.8 ±177.8
	Portal vein	FMN	A_1 0.01748 ±0.00283	α_1 0.3211 ±0.0462	0.04948 ±0.00206	V_1 54.3 ±7.6
			A_2 0.001337 ±0.000427	α_2 0.02241 ±0.00097		V_2 157.4 ±20.9
		Weight function	D_3 0.04304 ±0.00381	α_3 0.1816 ±0.0489	—	V_3 173.0 ±12.5
			D_4 0.001152 ±0.000201	α_4 0.02457 ±0.00744		V_4 188.9 ±32.5
10 μ mol FMN	Femoral vein	FMN	A_1 0.1761 ±0.0248	α_1 0.3574 ±0.0357	0.05243 ±0.00238	V_1 54.4 ±8.3
			A_2 0.01567 ±0.00124	α_2 0.02512 ±0.00146		V_2 160.9 ±14.3
		Weight function	D_3 0.06940 ±0.01338	α_3 0.1800 ±0.0403	—	V_3 125.6 ±34.1
			D_4 0.01024 ±0.00443	α_4 0.01867 ±0.00707		V_4 107.1 ±45.9
	Portal vein	FMN	A_1 0.1852 ±0.0138	α_1 0.3987 ±0.0379	0.06899 ±0.00864	V_1 48.8 ±3.3
			A_2 0.02176 ±0.00192	α_2 0.03003 ±0.00378		V_2 133.5 ±15.1
		Weight function	D_3 0.07448 ±0.00928	α_3 0.1618 ±0.0336	—	V_3 102.1 ±1.11
			D_4 0.01013 ±0.00417	α_4 0.02292 ±0.00867		V_4 163.1 ±50.9
0.56 μ mol FR	Femoral vein	FR	A_3 0.005217 ±0.000470	α_3 0.3240 ±0.0335	—	V_3 86.8 ±7.4
			A_4 0.001361 ±0.000098	α_4 0.02684 ±0.00724		V_4 164.4 ±30.6

Coefficient: μ_M for A_1 and A_2, dimensionless for A_3 and A_4. Rate Coefficient: min^{-1}. Distribution volume: ml. K_2: min^{-1}. Data are averages of three experiments with (±) standard error. A_3 and A_4 are coefficient of the two exponential formula for the FR plasma level after injection of FR itself.

The distribution volume of central compartment of FR is estimated from the following calculation. Sum of D_3 and D_4 in equation 22 is expressed as follows, when k_{24} is neglected.

$$D_3 + D_4 = \frac{V_1}{V_3}\left[\frac{(K_2-\alpha_3)(K_4-\alpha_3)k_{13}}{(K_2-\alpha_3)(\alpha_4-\alpha_3)} + \frac{(K_2-\alpha_4)(K_4-\alpha_4)k_{13}}{(K_2-\alpha_4)(\alpha_3-\alpha_4)}\right] \quad (24)$$

From the rearrangement, V_3 is expressed as follows,

$$V_3 = \frac{V_1 k_{13}}{D_3 + D_4} \tag{25}$$

where V_1 is the distribution volume of the central compartment of FMN and it is calculated as the ratio of the dose of injected FMN to sum of A_1 and A_2 that are the coefficients of the two exponential formula for FMN plasma level. The rate coefficients with negative sign, $-\alpha_1$ and $-\alpha_2$, are the roots of quadric equation 16 and then the next relationship between root and coefficient is expressed.

$$\alpha_1\alpha_2 = K_1K_2 - k_{12}k_{21} \tag{26}$$

when k_{24} is neglected, K_2 is equal to k_{21} and the next is expressed.

$$\alpha_1\alpha_2 = K_2(K_1 - k_{12}) \tag{27}$$

Since K_1 is sum of k_{12}, k_{13}, and k_{10} in the model shown in Fig. 2, the following is expressed.

$$\frac{\alpha_1\alpha_2}{K_2} = k_{13} + k_{10} \tag{28}$$

From equations 25 and 28, the next unequal expression is given.

$$V_3 \leqq \frac{V_1\alpha_1\alpha_2}{(D_3 + D_4)K_2} \tag{29}$$

The equal sign in above expression is valid when k_{10} is neglected.

The values of V_3 listed in Table III for FR after the injection of FMN are calculated from the assumption of negligible k_{10}. From the comparison between this values in the dose of 1 μmol FMN and the distribution volume obtained directly from FR plasma level after the injection by itself, the validity of unequality in equation 29 is considered to be evident. Consequently, the unequality suggests the existence of k_{10} which means the elimination of FMN other than metabolism to FR. And this result supports the explanation of the route dependent AUC of FR after ingestion of FMN by the linear compartment theory described before.

The values of V_3 after injection with the dose of 10 μmle FMN is less than those with the dose of 1 μmol. The comparison of V_3 to the direct observation after the injection of 0.56 μmol FR would not be adequate in the cases of 10 μmol FMN inejction, since the plasma level of FR after 10 μmol FMN injection is sufficiently higher than that after 0.56 μmol FR injection. For more concrete discussion, however, the investigation should be necessary by the non-linear pharmacokinetics.

The plasma level of FR after the injection of FMN is expected to be reconstituted from the convolution between both two exponential formula corresponding to the FMN plasma level and the weight function. The dotted line in Fig. 4 is calculated by the convolution with the mean parameters of three experiments. The observed FR plasma level is obviously lower than the calculated line. This discrepancy is presumed to be caused by the approximation of the two exponential curve to the weight function, since the numerical calculation used in

Table IV. Model Dependent Rate Constants (min^{-1}) and Lag Time (min)

Dose	Route	k_{12}	$k_{21} \times 10^{-1}$	$k_{13}+k_{14}+k_{10}$	$k_{31} \times 10^{-1}$	$k_{43} \times 10^{-1}$	k_{30}	lag time (min)
1 μ mol FMN	Femoral vein	0.2719 ±0.0379	0.7439 ±0.0723	0.1990 ±0.0122	0.6764 ±0.2295	0.4286 ±0.0257	0.09342 ±0.01147	0.91 ±0.02
	Portal vein	0.1493 ±0.0264	0.4949 ±0.0206	0.1447 ±0.0192	0.4690 ±0.2788	0.3962 ±0.1798	0.1197 ±0.0134	1.08 ±0.23
10 μ mol FMN	Femoral vein	0.1581 ±0.0142	0.5243 ±0.0224	0.1719 ±0.0214	0.4073 ±0.1193	0.5742 ±0.2349	0.1186 ±0.0196	1.37 ±0.19
	Portal vein	0.1866 ±0.0215	0.6899 ±0.0864	0.1731 ±0.0137	0.4971 ±0.1072	0.3938 ±0.1601	0.09495 ±0.01709	1.33 ±0.25

Data are averages of three rats with (±) standard error.

this study has been proved to be accurate enough.[13] A lag time is assumed for the correction of this discrepancy because of the simplification of calculation as follows,

$$\begin{aligned} &W(t) = 0 \quad 0<t\leqq DT \\ &W(t) = A_3\exp(-\alpha_3 t) + A_4\exp(-\alpha_4 t) \quad t>DT \end{aligned} \tag{30}$$

where W (t) is the weight function as the function of time t, DT is the lag time assumed newly, and A_3, A_4, α_3, and α_4 are the parameters in the two exponential formula. The time course of FR plasma level after FMN injection, C is expressed by the convolution with lag time as follows.

$$\begin{aligned} C &= \int_{TD}^{t} [\{A_3\exp(-\alpha_3\theta)+A_4\exp(-\alpha_4\theta)\}\cdot \\ &\quad \{A_1\exp(-\alpha_1(t-\theta))+A_2\exp(-\alpha_2(t-\theta))\}]d\theta \\ &= \frac{A_1A_3}{\alpha_3-\alpha_1}\{e^{-\alpha_1 t}\cdot e^{-(\alpha_3-\alpha_1)DT}-e^{-\alpha_3 t}\} + \frac{A_2A_3}{\alpha_3-\alpha_2}\{e^{-\alpha_2 t}\cdot e^{-(\alpha_3-\alpha_2)DT}-e^{-\alpha_3 t}\} \\ &+ \frac{A_1A_4}{\alpha_4-\alpha_1}\{e^{-\alpha_1 t}\cdot e^{-(\alpha_4-\alpha_1)DT}-e^{-\alpha_4 t}\} + \frac{A_2A_4}{\alpha_4-\alpha_2}\{e^{-\alpha_2 t}\cdot e^{-(\alpha_4-\alpha_2)DT}-e^{-\alpha_4 t}\} \end{aligned} \tag{31}$$

The solid line in Fig. 4 is calculated by equation 31 in which the value of DT is determined so as for the calculated C value to be just over the FR level observed at two minutes after the injection by means of the repeated calculation in 0.01 minute step of the increasing DT value. In every experiment, the observed FR plasma level is close to the calculated line with the correction of DT until 120 minutes and moreover the evaluated DT values are all less than two minutes as listed in Table III. Such a lag may suggest the existence of short time lag or quick rate process which is located in the intermediate between the elimination of FMN and the creation of FR, though the further investigation is needed to clarify the biological meanings. In Table IV, the pharmacokinetic parameters dependent on the model are listed from the usual calculation. The rate constants listed here are scarcely varied with difference of the injected dose and route. The dose dependency seen in the FR level is caused by change of the distribution volume but the rate constants. The decrease in FR total clearance is thought for example to come from the elevated level in plasma of FR by the saturation of binding between FR and tissue, though the experimental evidence has not been made yet.

Table V. Per Cent of FR Excreted in Bile and Urine in 24 hr

Dose	Route	Bile (%)	Urine (%)	Total (%)
1 μ mol FMN	Femoral vein	32.8±2.1	53.9±6.0	86.8±4.2
	Portal vein	31.1±0.7	58.8±1.2	90.0±1.9
10 μ mol FMN	Femoral vein	20.5±1.3	69.4±2.7	89.9±4.0
	Portal vein	22.2±1.9	64.9±2.2	87.1±2.8

Data are averages of three rats with (±) standard error.

Table V lists the per cent of FR excreted in urine and bile in 24 hours after injection. The excretion of FMN is not detectable in the present experiment, but it could not be neglected because of the instability of FMN in urine and bile. The recovery of FR in both urine and bile dose not reach 90 per cent of dose in 24 hours and the further recovery is hardly expected because of the short half life of FR. The existence of the other route of the excretion or metabolism in FR, therefore, could not be neglected completely. Since total recovery of FR does not vary with the difference of the dose, the dose dependency in plasma level of FR should not be thought to come from the variance in the metabolic ratio of FMN. The decreasing excretion ratio in bile with increasing the dose which is combined by the increasing urinary excretion is found in average but this is not significant statistically.

In the present study, FMN and FR pharmacokinetics is suggested to have rather complicated properties, but the route dependency of FR blood level after FMN ingestion, which is contrary to the first pass effect in liver, is successfully explained by the compartment model.