# Pharmacokinetic Profile of Flavin Adenine Dinucleotide, Flavin Mononucleotide and Riboflavin Following Intravenous Administration of Riboflavin or Its Coenzymes in Rats

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The pharmacokinetics and utilization (flavocoenzyme formation) of an intravenously administered flavin mononucleotide (FMN) or riboflavin (RF) in rats were assessed using ethylenediamine-N, N, N', N'-tetraacetic acid (EDTA)-treated plasma. Serial blood samples were collected via a syringe containing EDTA, used to prevent flavin adenin dinucleotide (FAD) hydrolysis, from the left jugular vein and plasma samples were analyzed for FAD, FMN and RF levels using a reversed-phase HPLC method. After the intravenous bolus administration of a single dose of FMN (500 nmol/kg), FAD was identified in plasma. However, increased levels of FAD in plasma were much less pronounced than that of RF levels. In contrast, after the intravenous bolus administration of single dose of RF (500 nmol/kg), both FAD and FMN levels were not different from the endogenous levels. Evidence is given in this paper that intravenously administered FMN affects the plasma levels of FAD in rats, whereas insensible changes in FMN and FAD levels were not confirmed by intravenously administered RF. The plasma kinetics of total flavin after intravenous bolus administration of RF, FMN or FAD to rats was also estimated. Statistical analysis of individual data revealed a high correlation between the total flavin level obtained by summation of each flavin level and that obtained with the lumiflavin (LF) method. Since this method is highly sensitive, it could be used to give precise values for the pharmacokinetic parameters of flavins.

Key words — riboflavin, flavin mononucleotide, flavin adenin dinucleotide, ethylenediamine-N, N, N', N'-tetraacetic acid, lumiflavin method, rat pharmacokinetics

#### INTRODUCTION

Riboflavin (RF) is a water-soluble vitamin that serves as a precursor for flavin adenin dinucleotide (FAD) and flavin mononucleotide (FMN). These compounds are involved in several reductionoxidation reactions and take part in the metabolism of folate, vitamin  $B_{12}$ , and other vitamins, and this probably explains why plasma RF is a determinant of plasma homocysteine, which is associated with cardiovascular disease, pregnancy complications, and cognitive impairment.<sup>1)</sup>

Simultaneous determinations of RF, FMN and FAD in plasma samples have not been widely used for pharmacokinetic studies of orally and intravenously administered vitamin B<sub>2</sub> (VB<sub>2</sub>) in animals and humans. Partly, this has been due to the lack of a suitable method. The instability of FAD and FMN in rabbit plasma was reported by Okumura and Yagi.<sup>2)</sup> Because of the rapid hydrolysis of FAD in human plasma after sample collection, Zempleni et al. determined flavocoenzymes in total as FMN by acid hydrolysis.<sup>3-5)</sup> We also previously reported that hydrolysis rates of FAD in rat and human plasma were very rapid.<sup>6)</sup> In order to overcome this difficulty, we investigated an effective extraction method of flavins in plasma without hydrolysis of FAD and FMN. Hydrolysis of FAD was markedly inhibited when ethylenediamine-N, N, N', N'-tetraacetic acid (EDTA) was added to the plasma.<sup>7)</sup> Simultaneous measurement of RF, FMN and FAD should be possible using plasma pretreated with EDTA. Therefore, we attempted to obtain real plasma concentrations after an intravenous administration of 500 nmol/kg as FAD to rats.

This study examined the pharmacokinetics of RF, FMN and FAD measured by adding excess EDTA, an inhibitor of FAD hydrolysis, in plasma under strict light and temperature control after intravenous administration of 500 nmol/kg as FMN or RF to rats. In addition, we assessed whether the sum of each flavin data obtained with our assay proce-

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dure correlated with total flavins obtained with the Lumitlavin HPLC method, the commonly used index to monitor  $VB_2$ .

#### MATERIALS AND METHODS

Materials — FAD was purchased from ICN Biomedical, Inc. (Aurora, OH, U.S.A.) and FMN, RF and EDTA (C<sub>10</sub>H<sub>14</sub>N<sub>2</sub>Na<sub>2</sub>O<sub>8</sub>.2H<sub>2</sub>O) were obtained from Kanto Chemicals (Tokyo, Japan). Galactoflavin (GF) was kindly gifted from Merck & Co., Inc. (Rahway, NJ, U.S.A.). LF was obtained from Sigma Chemicals (St. Louis, MO, U.S.A.). Injectable riboflavin sodium phosphate solution and hydroxypropyl- $\beta$ -cyclodextrin were purchased from Nippon Iyakuhin Kogyo (Toyama, Japan) and Junsei Chemical Co., Ltd. (Tokyo, Japan), respectively. Other chemicals were of reagent or HPLC grade. Measurement of Stability in Plasma, Intestinal **Mucosal Cells and an S9 Supernatant Fraction** from the Liver — Male GALAS rats (BrlHan: WIST@Jcl) at eight weeks of age were gifted by CLEA Japan, Inc. (Tokyo, Japan) and kept in standard cages with free access to water and standard chow. Animal care was performed according to the Standards Relating to the Care and Management of Experimental Animals and the guidance established by the ethical committee for Experimental Animals of Faculty of Pharmaceutical Science, Josai International University.

Rats were fasted for 18 hr before sacrifice and decapitated. Plasma previously prepared using heparin was stored below  $-30^{\circ}$ C. The small intestine and liver was removed promptly and rinsed with cold saline. The mucosal tissue was scraped off gently with a glass slide at 4°C. Cellular scrapings were weighed and homogenized for 30 s with iced 0.1 M Tris buffer, pH 8. The mucosal extracts were then centrifuged at  $60000 \times g$  for 30 min, and the supernatant solution was decanted and used to measure the hydrolysis of each flavin.<sup>8)</sup> The livers were also weighed and homogenized in four volumes of 0.15 M potassium chloride. The homogenate was centrifuged at 9000  $\times g$  for 20 min, and the supernatant fraction (S9 fraction) was removed and diluted in the same solution to yield a final concentration in the assay tubes equivalent to 1 mg/ml wet weight of hepatic tissue.<sup>9)</sup> All steps were carried out at 0°C.

FAD, FMN and RF were dissolved in distilled water to make a 1 mmol/l solution. The experi-

ments were initiated by adding the flavin solution to produce a final concentration of 1200 nmol/l (*ca.* 1 µg/ml). Each flavin was incubated for 10 min at 37°C in 20 volumes of the standard incubation mixture containing the plasma, the 60000 × g supernatant fraction from the intestinal mucosa or the 9000 × g supernatant fraction from the liver. A 60 µl aliquot of ice-cold methanol was added to each 20 µl sample immediately after it was taken from the reaction mixture. The sample was then mixed with 120 µl ice-cold water, and cooled in an ice-bath. The mixture was stirred in a vortex mixer for 5 s and centrifuged at 10000 × g for 1 min; 30 µl supernatant was injected into the HPLC. All operations were carried out under low-intensity light.

Animal Study —— RF was dissolved in 20% hydroxypropyl- $\beta$ -cyclodextrin solution. Injectable riboflavin sodium phosphate was used as an FMN parenteral formulation. Each VB<sub>2</sub> formulation was intravenously administered at a dose of 500 nmol/kg by bolus injection into the tail vein. Test tubes and syringes were previously placed on ice before sampling. At predetermined post-administration times, blood samples were withdrawn from the left jugular vein of the rats into syringes containing EDTA. EDTA-treated plasma was obtained by collecting blood into centrifuge tubes, giving a final EDTA concentration of 1 mmol/l. The samples were immediately placed on ice and centrifuged at  $0^{\circ}$ C to separate plasma (10000 × g, 1 min). The plasma was then processed further. Sample handling was carried out under dim light to avoid photodegradation of the B<sub>2</sub>-vitamers assayed.

**Sample Analyses** — These plasma samples were analyzed for FAD, FMN and RF using GF as an internal standard (IS) by HPLC with a fluorescence detector, as described previously.<sup>6)</sup> In a 1 ml test tube, 60 µl ice-cold methanol was added to 20 µl plasma to precipitate proteins. IS solution was added to the tube, it was vortex-mixed for 1 min and then centrifuged for 1 min at 10000 × *g* to separate proteins. The supernatant was diluted by addition of the same volume of 10 mmol/l KH<sub>2</sub>PO<sub>4</sub> and 30 µl supernatant was used for the HPLC run.

The concentration of total VB<sub>2</sub> in plasma was estimated by the LF-HPLC method.<sup>10)</sup> Briefly, after plasma deproteination, a photolysis step in alkaline solution by irradiation with a fluorescent light was performed to convert more photo-labile FAD, FMN and RF into photo-stable LF. Plasma was mixed with 0.5 mol/l H<sub>2</sub>SO<sub>4</sub> and heated in a water bath 80°C for 15 min. After deproteinizing by 10% trichloroacetic acid, the supernatant was obtained by centrifugation  $(3000 \times g, 10 \text{ min})$ . An equal volume of a solution of 1 mol/l NaOH was added to each sample. Under the alkaline condition, LF was produced by shedding light (100 W, 20 cm, 1 hr) and subsequently pH was adjusted to neutral by adding acetic acid. Each aliquot was filtered through a filter (0.45 µm)(Millex-HV, Millipore Corp., Billerica, MA, U.S.A.) and injected into a HPLC system.

Chromatographic Conditions — As reported by Capo-chichi et al.,<sup>11)</sup> routine analysis of the plasma B2-vitamers was carried out on a C18 reversed-phase column  $(250 \times 4 \text{ mm}, 5 \mu \text{m})$ (LichroCART250-4 RP18), which was kept at 40°C with an isocratic eluent (10 mmol/l KH<sub>2</sub>PO<sub>4</sub>(pH 5.0):CH<sub>3</sub>OH=3:1) at a flow-rate of 0.8 ml/min. The eluent was filtered through a 0.45 µm membrane (DURAPORE, Millipore Corp., Billerica, MA, U.S.A.) and degassed under a vacuum prior to use in HPLC. The HPLC system was composed of LC-10ADvp pumps connected to an RT-10AxL fluorescence monitor and a CTO-10ACvp column oven (both from Shimadzu, Kyoto, Japan). The spectro-fluorometer was set at 440 nm for the excitation wavelength and 560 nm for the emission wavelength. The retention times were fairly reproducible and the recoveries of FAD, FMN, RF and LF added to plasma samples were 94.4±8.6, 95.2±4.1, 100.9±2.7 and 87.1±5.8%, respectively. Data Analysis — Pharmacokinetic analysis of the plasma concentration data was performed using model-independent methods.<sup>12)</sup> Area under the plasma concentration-time curve from zero to infinite time (AUC), total plasma clearance (CL), mean residence time (MRT) and apparent volume of distribution at steady-state (Vdss) were calculated. AUC was calculated according to the trapezoidal rule. The plasma elimination half-life  $(T_{1/2})$  and plasma concentration at time 0  $(C_0)$ were determined by least-squares regression analysis of terminal and distribution log-linear portions of the plasma concentration-time profile, respectively. The peak plasma metabolite concentrations (*Cmax*) are provided as recorded and not by extrapolation of theoretical values. MRT was calculated using statistical moment theory<sup>12)</sup> and CL was determined according to the following equation:

CL = D/AUC

where D is the dose. The square of correlation coefficients was determined by least-squares linear regression analysis. A *p*-value of less than 0.05 was considered statistically significant correlation.

### **RESULTS AND DISCUSSION**

### **Stability of Flavins in Rat Enzymatic Fractions**

Hydrolysis of FAD, FMN and RF after 10 min at 37°C in plasma, liver S9 fraction and intestinal mucosal cell fractions were measured using HPLC, as shown in Fig. 1. Rapid decomposition of FAD was found to take place in plasma obtained from heparinized- blood specimens as previously described.<sup>7)</sup> In contrast, FMN was hydrolyzed slowly in the plasma, and the residual percentage was 95% or more at 10 min, and about 87% at 30 min, at 37°C, respectively. No decrease in RF concentration was observed after incubation of RF with the plasma.

Almost all RF in tissues is enzyme-bound.<sup>1)</sup> Unbound coenzyme forms are relatively labile and are rapidly hydrolyzed to free RF, which diffuses from cells and is excreted.<sup>1)</sup> EDTA has been shown to inhibit the activity of enzymes that hydrolyze FAD and FMN. Therefore, the plasma was separated immediately after obtaining the blood samples,<sup>2)</sup> and a large excess of EDTA was used as an antico-



Fig. 1. Hydrolysis of Flavins by Rat Plasma, Liver S9 Fraction and Intestinal Mucosal Fraction at 37°C for 10 min Each data represents the mean ± S.D. of three specimens.

agulant to avoid determining artificially enhanced RF and reduced FAD and FMN. The activity of enzymes that hydrolyze FAD and FMN has been shown to inhibit under these conditions as reported previously.<sup>6)</sup> No less than 95% of FAD and FMN in EDTA-treated plasma remained at 30 min at 4°C (data not shown).

The hydrolysis of FAD and FMN were also found to take place in both hepatic S9 fractions and intestinal mucosal preparations isolated from rats. The rates of hydrolysis of FMN by the liver and intestinal mucosa were higher than those of FAD *in vitro*. Although percentage RF remaining exceeded 100% substantially in intestinal mucosal fraction, it seemed like RF was stable in all preparations as illustrated in Fig. 1. We could not exclude that metabolism by rat liver and small intestine played some role in this hydrolysis after the administration of FAD or FMN.<sup>13,14</sup> These results also suggest that B<sub>2</sub>-vitamers may be preferentially transferred from the liver and small intestine to the plasma as RF.

#### Pharmacokinetics of FMN and RF in Rats

Endogenous plasma concentrations of FAD, FMN and RF in rats were [median (range)] 29.6 (20.5–48.4), 46.7 (25.0–81.7) and 42.4 (32.0– 55.0) nmol/l, respectively. The plasma concentrations of endogenous flavins were subtracted from plasma concentrations after intravenous administration. Figure 2 shows the plasma concentrations of FAD, FMN and RF after intravenous administration of FMN or RF at 500 nmol/kg in rats. The dose was in accordance with a human pharmacokinetic trial of VB<sub>2</sub>.<sup>7)</sup>

Plasma levels of FMN appeared to exponentially decrease with time. The pharmacokinetic parameters for FAD, FMN and RF are summarized in Table 1. The elimination rate of FMN was very rapid, which was reflected in the small value of  $T_{1/2}$  (2.1 min) and in the large value of CL (0.13 l/min/kg). The AUC for FMN was 4257.1±1403.0 nmol.min/l. FAD and RF were also detected in plasma, and the AUC values for these compounds were 1367.9 and 25533.7 nmol.min/l, respectively. Rapid conversion of FMN to RF was observed immediately after intravenous administration of FMN in rats, unlike what was observed in the in vitro study using rat plasma (Fig. 1). These results suggest that FMN in the plasma samples was not fully degraded, and that the rat metabolic organs such as liver and small intestine can metabolize FMN externally administered (Fig. 1).

Interestingly, a detectable amount of FAD was also found in rat plasma samples after the intravenous bolus injection of FMN. The experimental findings reported by Barile et al. show that FAD synthesis derived directly from externally added FMN occurred in isolated rat liver mitochondria.<sup>15)</sup> However, the detection of FAD partially disagrees with a previous report that used plasma obtained from a human subject.<sup>16)</sup> The apparent discrepancies between our results and those published by Lopez-Anaya and Mayersohn might be attributed in part to different anticoagulants, infusion rates, storage conditions or animal species.<sup>16)</sup> In order to obtain reliable data, additional pharmacokinetic studies will be required under better experimental conditions in the future.

The plasma levels of RF after the intravenous



Fig. 2. Plasma Concentration of FAD (●), FMN (○), and RF (△) vs. Time Curves after Intravenous Administration of FMN (a) or RF (b) at 500 nmol/kg in Rats

Each point represents the mean  $\pm$  S.D. of four determinations

Compound	Р	arameter		RF			MN	FAD	
FMN	$C_0$	(nmol/l)				$1104.5 \pm 300.1$		300.1	_
	Cmax	(nmol/l)	1569.9	± 29	99.9				$196.4 \pm 57.6$
	AUC	(nmol.min/l)	25533.7	$\pm 854$	46.4	4257.1	$4257.1 \pm 1403.0$		$1367.9 \pm 449.7$
	CL	(l/min/kg)		_		0.13	±	0.03	
	MRT	(min)				3.1	±	0.4	
	Vdss	(l/kg)				0.39	±	0.10	
	$T_{1/2}$	(min)	55.4	± 1	$17.0^{a}$	2.1	±	0.6	$4.5 \pm 3.2$
RF	$C_0$	(nmol/l)	1906.3	± 37	77.0				
	Cmax	(nmol/l)		_					
	AUC	(nmol.min/l)	24469.6	± 612	21.9				
	CL	(l/min/kg)	0.02	2 ±	0.010				
	MRT	(min)	43.5	±	5.7				
	Vdss	(l/kg)	0.94	±	0.22				_
	$T_{1/2}$	(min)	49.6	±	9.8 <sup><i>a</i>)</sup>				—

Table 1. Pharmacokinetic Parameters of FAD, FMN and RF after Intravenous Administration of FMN or RF at 500 nmol/kg in Rats

Each value represents the mean  $\pm$  S.D. of four rats. *a*) Half-life of terminal phase.



Fig. 3. Sum of Flavins (o: FAD+FMN+RF) and LF (o: Total Flavins) Plasma Concentrations as a Function of Time after Intravenous Administration of FAD (a), FMN (b) or RF (c) at 500 nmol/kg in Rats

Each point represents the mean  $\pm$  S.D. of four determinations. As for FAD (a), each flavin level was calculated from the data from our recent study.<sup>6)</sup>

administration of RF appeared to decrease with time in a biexponential pattern, *ie*, a rapid phase of elimination was followed by a slower terminal phase. Changes in the concentrations of RF were almost the same compared with those after the intravenous administration of FMN. Therefore, the *AUC* and terminal  $T_{1/2}$  values for RF were 24469.6 nmol.min/l and 49.6 min which agreed with the values after the administration of FMN, 25533.7 nmol.min/l and 55.4 min, respectively. The *CL* and *Vdss* of RF, as calculated from the intravenous RF administration, were 22 ml/min/kg and 0.94 l/kg. On the other hand, increased levels of FAD and FMN were not analytically detectable in plasma samples after the intravenous administration of RF. This is in agreement with data published by Zempleni *et al.*, who found

that the response of total flavocoenzymes in plasma was much less pronounced than that of RF in humans.<sup>7)</sup>

# Comparative Determination of Total Flavins in Rat Plasma by Direct Measurement of Each Flavin and LF Method

The LF method for estimating total VB<sub>2</sub> in biological fluid is widely used because B<sub>2</sub>-vitamers are highly photosensitive compounds.<sup>10)</sup> LF, a major photoproduct, was assayed by a fluorescence method using HPLC. The baseline level of LF and the sum of three flavins in rat plasma before the administration were [median (range)] 135.8 (101.9-185.4) and 126.2 (108.2-149.9) nmol/l, respectively. The total flavin level obtained as LF was in good agreement with that obtained by summation of each flavin level for the same plasma extract (Fig. 3). With regard to FAD, each flavin level was calculated from the data from our recent study.<sup>6)</sup> The values established by the LF method were slightly lower. This underestimation might be partially due to the low recovery rate of LF from the plasma. Several studies suggested deproteinization by 10%-trichloroacetic acid as a possible cause of low recovery rates.<sup>17,18)</sup> However, the relationship between total flavin level obtained as LF and the sum of the three flavins is shown in Fig. 4, and a statistically close correlation can be seen ( $R^2$  = 0.9647, p < 0.01). As can be seen in Figs. 3 and 4, the present procedure for direct extraction of flavins without hydrolysis of FMN and FAD gave satisfactory results as compared with the LF fluorescence





Fig. 4. Correlation Graph between Sum of Flavins (FAD+FMN+RF) and LF (Total Flavins) Levels in Rat Plasma

Dose: 500 nmol/kg of FAD (•), FMN (•) and RF ( $\triangle$ ). y = 0.8689x + 9.9878 (n = 102),  $R^2 = 0.9647$ .

method in the plasma. In addition, the mean values of  $T_{1/2}$  for LF were 44.6±13.0, 44.3±19.6 and 50.8±23.4 min after intravenous administration of RF, FMN and FAD, respectively, which agreed with those for RF.

It should be emphasized that the present assay procedure enabled a more precise determination of the content of each flavin in plasma after externally administered flavins. This method is not simple, but is more sensitive, and could be adapted for use with other biological fluids.

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