〈Brief Note〉

Interaction of warfarin with enteral formulas and their protein components *in vitro*

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Summary Simultaneous administration of enteral formula and warfarin in the clinical setting can shorten or extend the prothrombin time-international normalized ratio (PT-INR). We previously found that the free warfarin rate is reduced when warfarin and enteral formulas (Mei Balance R®, Mei Flow®, F2 Light®, and PG Soft EJ®) are mixed *in vitro*. In this study, we examined the binding between warfarin and enteral formulas or their protein components by quantifying the free warfarin concentration using HPLC and analyzed the binding site. The whey protein Lactocrystal® showed the lowest free warfarin rate of the various mixtures of warfarin and proteins contained in the enteral formulas. A Scatchard plot revealed two binding sites with warfarin in four types of enteral formulas and three types of whey proteins (Lactocrystal®, PROGEL800®, and Wheyco W80®). These results showed that warfarin and the proteins in enteral formulas bind to each other, which may inhibit the absorption of warfarin from the small intestine. When the enteral formula type is changed during the co-administration of warfarin and enteral formula, precautions need to be taken, such as monitoring of warfarin or PT-INR.

Key words: Warfarin, Enteral formulas, Protein, Scatchard plot, Ultrafiltration

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1. Introduction

The anticoagulant warfarin has been used worldwide for decades. However, many interactions related to vitamin K and the drug-metabolizing enzyme CYP2C9 have been reported[1-3]. Therefore, attention must be paid to food intake and medication use. Warfarin is used to treat and prevent thromboembolism and is often used in combination with enteral formula in critically ill patients. However, simultaneous administration of warfarin with enteral formula has been reported to shorten the prothrombin time-international normalized ratio (PT-INR), which is an indicator of warfarin efficacy[4], or to reduce the maximum serum warfarin concentration[5]. One reason for this is that the vitamin K in enteral formulas antagonizes the inhibitory effect of the warfarin on the biosynthesis of vitamin K-dependent coagulation factors[4]. However, even if the vitamin K content in the enteral formula is set to a dose that does not affect the anticoagulant properties, the effect of the warfarin is weakened with co-administration of warfarin and enteral formula[6]. Therefore, these factors are considered to reduce warfarin activity.

The binding rate of warfarin to plasma protein is very high, from 90% to 99%[7], but it only exerts pharmacological effects in its free form[7]. Therefore, the proteins in enteral formula may also contribute to the weakening of warfarin action[6]. According to Penrod *et al*.[4], several enteral formula products bind warfarin, reducing its bioavailability. In the clinical setting, changing the enteral formula from Mei Balance R® to F2 Light® shortens PT-INR, while further changing to Mei Flow® extends PT-INR to within the recommended treatment range[6]. When daily vitamin K intake exceeds 250 μg, the probability of adverse events (a shortened PT-INR) increases[8]. In Sato *et al*.[6], vitamin K intake was less than 250 μg, so the authors ruled out an effect of vitamin K. Furthermore, when warfarin was added to the enteral formulas, the free warfarin rates measured by HPLC were 66.6% for Mei Balance R®, 44.2% for Mei Flow®, and 12.6% for F2 Light®[6]. Based on their findings, we studied the binding between warfarin and these enteral formulas or their protein components by quantifying the free warfarin concentration and then performed a basic analysis of the binding site.

2. Materials and Methods

Materials

Warfarin sodium (biochemistry grade), methanol (HPLC grade), and phosphoric acid (guaranteed reagent grade) were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Mei Balance R® and Mei Flow® (Meiji Company Limited, Tokyo, Japan) and F2 Light® and PG Soft EJ® (TERUMO Company Limited, Tokyo, Japan) were obtained from commercial sources. The components of these enteral formulas are shown in Table 1. Milka MPI®, LE80GF-US®, Casein calcium S (-Ca)®, Lactocrystal®, PROGEL800®, Wheyco W80®, LACTOMIN 80-S®, and Willpro P20® were purchased from Nippon Shinyaku Company Limited (Kyoto, Japan). A Centrifree® centrifugal ultrafiltration device with a 30-kDa molecular weight cutoff was obtained from Merck Millipore (Tokyo, Japan). Human plasma (pool, heparin) was purchased from Tennessee Blood Service (Memphis, TN).

Instruments

Ultrafast liquid chromatography analysis was performed on a chromatographic system (Model No. CBM-20A, Shimadzu, Kyoto, Japan) consisting of a degasser (DGU-20A5), quaternary pump (LC-20AD), autoinjector (SIL-20A), column oven (CTO-20A), and dual-wavelength diode array detector (SPD-M20A). The analytical column used was an Inert Sustain™ C_{18} (4.6 × 150 mm; particle size, 5 μm; GL Sciences Inc.). Centrifugation was performed with an LC-230 centrifuge (Tomy Industries Company Limited, Tokyo, Japan).

HPLC conditions for determining warfarin concentrations

Free warfarin concentrations were measured using HPLC according to the test conditions for

Table 1 List of the main components in each enteral formula per 100 mL

		Mei Balance R®	Mei Flow®	F2 Light®	PG Soft EJ®
Calorie (kcal)		67.1	179.6	79.6	164.8
Protein	Total amount (g)	2.7	7.2	3.2	6.6
	Composition	Milk protein Soy protein Casein sodium	Milk protein Casein sodium	Whey protein Soy protein	Whey protein Soy protein
Lipid (g)		1.9	5.0	1.8	3.6
Carbohydrate (g)		10.5	28.6	13.6	26.5
Available carbohydrate (g)		9.9	25.9	12.3	25.9
Dietary fiber	Total amount (g)	0.7	2.7	1.3	0.6
	Composition	Liquid dextrin Indigestible dextrin Stabilizer (Carrageenan)	Dextrin Indigestible dextrin Soy fiber Thickener (Carrageenan)	Dextrin Soy fiber Agar	Dextrin Agar
Ash (g)		0.6	1.3	0.7	1.1
Water (g)		89.5	71.9	87.5	72.0
Vitamin K (μg)		3.4	11.3	11.9	24.7
pH		7	7	4	4

warfarin potassium tablets as established by the Japanese Pharmacopoeia[9]. HPLC was performed under the following conditions: mobile phase, 70% MeOH:phosphoric acid (1000:1); flow rate, 0.8 mL/min; column temperature, 35°C; injection volume, 10 μL. HPLC eluates were monitored by UV absorbance at 283 nm.

Scatchard plot of warfarin binding to plasma or enteral formula

A 495-μL plasma sample was added to a 5-μL aqueous solution of warfarin (10, 16, 20, 30, 32, 50, 62.5, 80, and 100 mg/mL), and a 1-mL sample of enteral formula was added to a 60-μL aqueous solution of warfarin (10, 30, 50, 80, 100, 300, 500, 800, and 1000 μg/mL). The protein concentration in the enteral formulas was set to 2 mg/mL to avoid the Donnan effect and protein–protein interactions[10]. These samples were incubated for 30 min at 37°C. Then, 80 μL of the plasma sample or 1060 μL of the enteral formula sample was pipetted into a Centrifree tube. After centrifugation (2000 × *g* for 10 min at room temperature), the concentration of ultrafiltrate (free) warfarin was quantified by HPLC[11]. A Scatchard plot was created from the obtained free warfarin concentration, and the binding site with warfarin was estimated by the formula r / [Df] = nK – rK, where r is the molar ratio of bound warfarin to protein, [Df] is the molar concentration of free warfarin at equilibrium, K is the association constant, and n is the number of binding sites[10].

Warfarin binding to whey proteins and its Scatchard plot

A 1-mL aqueous solution of eight types of proteins (50 mg/mL) was added to a 60-μL aqueous solution of warfarin (50 mg/mL). The protein concentration was set to the protein content of the enteral formula. These samples were incubated for 30 min at 37°C. After centrifugation (2000 × *g* for 10 min at room temperature), the concentration of free warfarin was quantified by HPLC[9].

We derived a Scatchard plot by adding a 1-mL aqueous solution of protein (Lactocrystal®, PROGEL800®, and Wheyco W80®) (2 mg/mL to avoid the Donnan effect and protein–protein interactions[10]) to a 60-μL aqueous solution of warfarin (10, 30, 50, 80, 100, 300, 500, 800, and 1000 μg/mL). The subsequent procedure was the same as the above.

Data analysis

Data are expressed as the mean ± standard error of the mean. Statistical analysis was performed using the statistical software package EZR, version 1.40[12]. The free warfarin rate was analyzed using the Tukey test. The relationship between the n·K value and the warfarin binding rate was estimated using Pearson's correlation test. All results with $p < 0.05$ were considered statistically significant.

3. Results and Discussion

Binding of warfarin in plasma and enteral formula

Based on our previous findings[6] of a decreased free warfarin rate when warfarin is mixed with enteral formula, we used a Scatchard plot to examine the binding of warfarin in the present study.

For plasma, the Scatchard plot of warfarin was curvilinear, suggesting the existence of two classes of binding sites (Fig. 1A). The association constant K of plasma closely agreed with that previously reported for serum[13] (Table 2). For four types of enteral formulas (Mei Balance R®, Mei Flow®, F2 Light®, and PG Soft EJ®), the Scatchard plot of warfarin was curvilinear, suggesting the existence of two classes of binding sites for all enteral formulas (Fig. 1B). The binding of warfarin to plasma was stronger than that to the enteral formulas because the number of binding sites (n) of plasma ($n_1 = 1.1 \times 10^3$, $n_2 = 2.7 \times 10^3$) was higher than that of the enteral formulas ($n_1 = 2.2$–7.4, $n_2 = 3.8 \times 10^1$–5.9×10^2) (Table 2). The binding constant K depended on the type of enteral formula. The lower free warfarin rates of F2 Light® and PG Soft EJ®[6] ($K_1 = 11.1$ and 7.1 μmol/L, $K_2 = 4.1 \times 10^2$ and 5.9×10^2 μmol/L) corresponded to a higher association constant K than for Mei Balance R® and Mei Flow® ($K_1 = 6.3$ and 5.2 μmol/L, $K_2 = 2.8 \times 10^2$ and 2.8×10^2 μmol/L). Our data suggested that F2 Light® and PG Soft EJ® exhibited stronger binding of warfarin than Mei Balance R® and Mei Flow®. This is likely to be caused by differences in the composition of these enteral formulas compared with F2 Light®–PG Soft EJ® and Mei

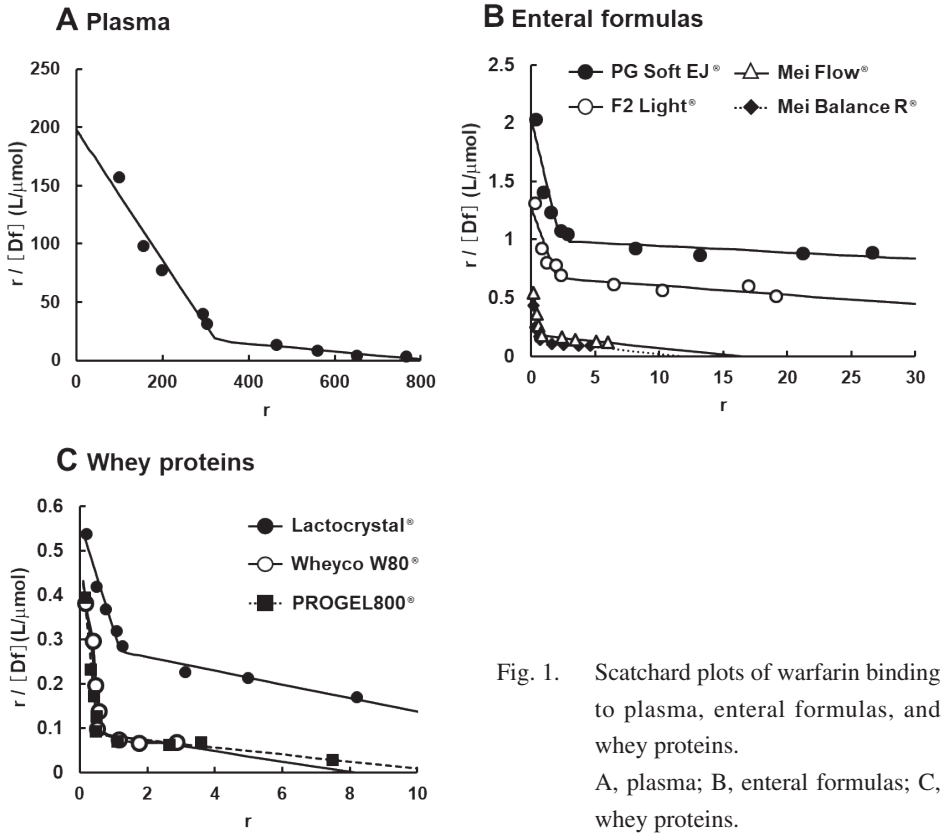

Fig. 1. Scatchard plots of warfarin binding to plasma, enteral formulas, and whey proteins.
A, plasma; B, enteral formulas; C, whey proteins.

Table 2 Binding parameters for the interaction of warfarin with plasma, enteral formulas, or whey proteins

Component	n_1	K_1 (μmol/L)	n_2	K_2 (μmol/L)
Plasma	1.1×10^3	6.2	2.7×10^3	9.6×10^1
Mei Balance R®	2.2	6.3	3.8×10^1	2.8×10^2
Mei Flow®	2.5	5.2	5.2×10^1	2.8×10^2
F2 Light®	6.6	11.1	2.8×10^2	4.1×10^2
PG Soft EJ®	7.4	7.1	5.9×10^2	5.9×10^2
Lactocrystal®	4.0	14.4	6.1×10^1	2.1×10^2
PROGEL800®	1.7	4.2	3.6×10^1	4.0×10^2
Wheyco W80®	2.2	5.4	2.6×10^1	2.8×10^2

n, number of binding sites; K, association constant

Balance R®–Mei Flow® (Table 1). Mei Balance R® and Mei Flow® contain casein, whereas F2 Light® and PG Soft EJ® do not. Milk protein comprises about 80% casein and 20% whey protein[14]. Whey is a byproduct of the production of cheese and other dairy products. It contains protein and sugar (lactose) and it can be valorized by the fermentative action of yeasts[15]. Therefore, it may be necessary to consider not only differences in the amount of protein but also changes in components such as lactose.

Binding of warfarin to whey and other types of proteins

Proteins of various origins were mixed with warfarin and the free warfarin rate was measured. Milka MPI®, derived from synthetic milk protein, had a free warfarin rate of 62.1 ± 1.0%, LE80GF-US®, derived from peptide protein, had a rate of 74.7 ± 1.3%, and Casein calcium S (-Ca)®, derived from casein protein, had a rate of 60.2 ± 4.8%. Lactocrystal®, PROGEL800®, Wheyco W80®, and LACTOMIN80-S®, all derived from whey protein, had free warfarin rates of 4.2 ± 0.2%, 75.3 ± 4.5%, 74.1 ± 5.6%, and 65.4 ± 10.6%, respectively (n = 3). Willpro P20®, derived from soy protein, had a free warfarin rate of 65.1 ± 3.3% (n = 3) (Fig. 2). Thus, the free warfarin rate decreased with all proteins, although Lactocrystal® had the lowest free warfarin rate of all protein types. Lactocrystal® is a whey-derived protein and whey protein is found in F2 Light® and PG Soft EJ® (Table 1). The reason for the strong effect of Lactocrystal® seems to be that it was a more acidic protein (pH 3.4) (product information for Lactocrystal® from Nippon Shinyaku Company Limited) compared with the other proteins (pH 6.5-7.0) (product information for Milka MPI®, LE80GF-US®, Casein calcium S (-Ca)®, PROGEL800®, and Willpro P20® from Nippon Shinyaku Company Limited). Since warfarin is an acidic medicine[16] and α_1-acid glycoprotein (pH 3) is reported to have an acidic ligand binding site, as well as a basic ligand binding site[17], Lactocrystal® may also interact with this acidic ligand binding site. It has been reported that β-lactoglobulin, a type of whey protein, binds to hydrophobic substances such as retinol, triglyceride, and long-chain fatty acids *in vitro*[18]. Moreover, HAMLET (human alpha-lactalbumin made lethal to tumor cells) is a complex of human α-lactalbumin, a type of whey protein, and oleic acid that induces apoptosis-like death in tumor cells but spares most healthy differentiated cells[19].

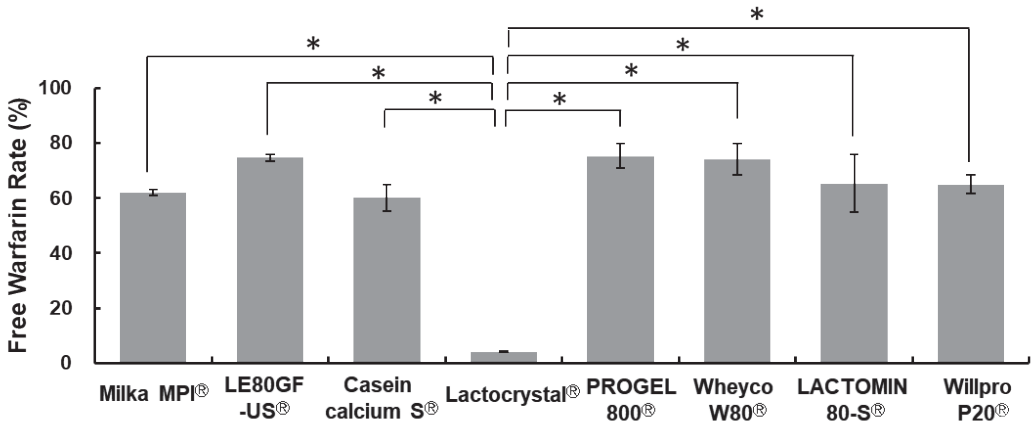

Fig. 2. Free warfarin rates for proteins mixed with warfarin.
Data are presented as the mean (bars) and SD (whiskers) of three independent experiments.
*$p < 0.01$ vs. Lactocrystal® by Tukey's test.

In addition, the Scatchard plots of whey proteins (Lactocrystal®, PROGEL800®, and Wheyco W80®) were curvilinear, suggesting the presence of two classes of binding sites (Fig. 1C). No difference in the association constant K was observed between Lactocrystal® (which showed the lowest free warfarin rate) and PROGEL800® and Wheyco W80® (which showed relatively high free warfarin rates) (Table 2). Furthermore, the binding rates of plasma, enteral formulas, and whey proteins with warfarin were significantly positively correlated with the n·K value (the n·K value is a parameter considered to represent the strength of the binding) (R = 0.947, $p = 1.14\times10^{-35}$) (Fig. 3). These results showed that a smaller n·K value was correlated with less binding of warfarin to protein and indicated a higher free warfarin rate, confirming the high correlation (R = 0.947) between the strength of the binding and the rates of warfarin binding to protein. These results showed that the protein in the enteral formulas and warfarin were bound to each other *in vitro* and the binding strength may be dependent on the type of protein or other components.

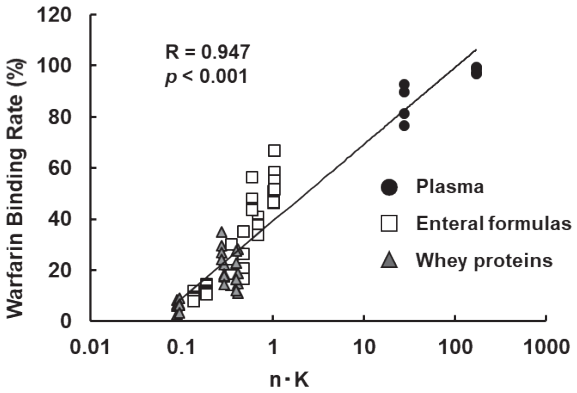

Fig. 3. Scatter plot of the warfarin binding rate against n·K.
R = 0.947, $p < 0.001$ by Pearson's correlation test.
n, number of binding sites; K, association constant.

4. Conclusion

Building on our previous findings[6], we have conducted further research into the binding of warfarin and enteral formulas. We found that a Scatchard analysis of the binding between enteral formulas and warfarin found that each enteral formula shows a different association constant K. Proteins with a large n·K value bind strongly to warfarin, suggesting that strong binding to protein might affect the absorption of warfarin from the intestine. Therefore, when the management of patients with warfarin and enteral formula is changed due to hospital transfer, PT-INR (an indicator of warfarin efficacy.) may be altered. When the enteral formula type is changed during the co-administration of warfarin and enteral formula, precautions need to be taken, such as monitoring of

warfarin or PT-INR.

Conflict of interest

The authors declare no conflict of interest.

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