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Improvement of physicochemical properties of N-4472 Part II: characterization of N-4472 microemulsion and the enhanced oral absorption

K. Itoh [a,b,*], S. Matsui [b], Y. Tozuka [a], T. Oguchi [a], K. Yamamoto [a]

[a] *Graduate School of Pharmaceutical Sciences, Chiba University, 1-33 Yayoicho, Inage-ku, Chiba 263-8522, Japan*
[b] *Nisshin Seifun Group Inc., 1-25, Kanda-Nishiki-cho, Chiyoda-ku, Tokyo 101-8441, Japan*

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Abstract

The optimized formulation of N-4472, *N*-[2-(3,5-di-tert-butyl-4-hydroxyphenethyl)-4,6-difluorophenyl]-*N′*-[4-(*N*-benzylpiperidyl)] urea, which was a poorly water-soluble drug, was developed by utilizing the complexation between N-4472 and L-ascorbic acid (VC). It was found that the formulation with Gelucire® 44/14, HCO-60® and sodium dodecyl sulfate provided a self-microemulsifying system consisting of fine droplets in approximately 18 nm size with a narrow distribution. ^{1}H-NMR spectroscopic study indicated that the N-4472/VC complex was molecularly incorporated into surfactant molecular assembly in the microemulsion droplets. It was found that the N-4472 microemulsion was stable at the pH range from 2.0 to 7.0, suggesting the stability in the gastrointestinal tract. When the microemulsion containing N-4472/VC complex was orally administrated in rats, high AUC value of N-4472 (2 to 4-fold) was observed in comparison with the aqueous solution containing N-4472/VC complex.

Keywords: Microemulsions; Solubilization; Oral absorption; Poorly water-soluble drug; NMR spectroscopy

1. Introduction

Microemulsion is a mixed system consisting of water, oil and surfactant, and is characterized as a transparent, optically isotropic and thermodynamically stable liquid (Danielsson and Lindman, 1981). Application of microemulsion as a drug carrier has been reported as a method of improving oral absorption of poorly water-soluble drugs (Kim et al., 1997, 2001; Gao et al., 1998; Khoo et al., 1998). With regard to the commercially available formulation of cyclosporin A, microemulsion formulation (Neoral®) is known to be superior to

* Corresponding author. Tel.: +81-43-290-2938; fax: +81-43-290-2939
E-mail address: itoh028@attglobal.net (K. Itoh).

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oil-based formulation (Sandimmune®) for improving oral absorption (Cooney et al., 1998). It is considered that the improved absorption from the microemulsion is due to incorporation of drug into microemulsion droplets, and the increased specific surface area resulted in enhancing the contact with the gastrointestinal tract (Shah et al., 1994). It is considered that the size of microemulsion droplets is one of the important factors relating to oral absorption. Another important factor is the inner polarity of droplets governed by the hydrophilic–lipophilic balance of surfactant. The change in the droplet polarity is considered to affect the arrangement of drug and surfactant on the droplet interface and to alter drug releasing property (Shah et al., 1994). Therefore, the molecular state of drugs and interaction mode between incorporated drug and surfactant should be clarified to obtain an optimized formulation of microemulsion.

Nuclear magnetic resonance (NMR) can be an excellent method to characterize the structure of microemulsion droplets. Since the linewidth of NMR signal is reflected by the mobility of the nuclei, the changes in the linewidth provide information on the state of drug molecules within the microemulsion droplets (i.e. associated or dispersed). Since the chemical shift of NMR signal is also reflected by the environment around the nuclei, the changes in the chemical shift provide information on interactions between the drug molecules and surfactant molecules within the microemulsion droplets (Rades and Mueller-Goymann, 1998; Jenning et al., 2000).

N-[2-(3,5-di-tert-butyl-4-hydroxyphenethyl)-4,6-difluorophenyl]-*N′*-[4-(*N*-benzyl-piperidyl)] urea (N-4472) is a newly developed drug with a lipid-lowering activity. As reported previously, the optimum self-microemulsifying formulation was developed in order to improve the aqueous solubility of N-4472, and this formulation formed stable microemulsion droplets in various aqueous media (Itoh et al., 2002a). In the present study, by using ^{1}H-NMR spectroscopy we estimated the physicochemical properties of microemulsion droplets which solubilized N-4472 (N-4472 concentration: 20 mg/ml) by using the carriers, Gelucire® 44/14, HCO-60® and SDS, and examined whether or not the oral absorption could be improved by microemulsification.

2. Materials and methods

2.1. Materials

N-4472 was synthesized by Nisshin Seifun Group Inc. (Japan). Gelucire® 44/14 (mixture of 30% glycerolester and 70% PEG-ester with fatty acids, Gattefosse Co., France) and polyoxyethylene (60) hydrogenated castor oil (HCO-60®, Nikko Chemicals Co. Ltd, Japan) were used as received. L-Ascorbic acid (VC) and sodium dodecyl sulfate (SDS) were of reagent grade and purchased from Wako Pure Chemical Industries, Ltd (Japan). Glycochenodeoxychoilc acid sodium salt (GCDCA-Na) was of reagent grade and purchased from Aldrich Chemical Co. Inc. (USA). Deuterium oxide (D_2O, Aldrich Chemical Co. Inc.), methanol-d_4 (CD_3OD, Euriso-top, France), sodium trimethylsilylpropanesulfonate (DSS, Aldrich Chemical Co. Inc.) and tetramethylsilane (TMS, Nacalai tesque, Inc., Japan) were of NMR reagent grade and were used as received. All other chemicals used were of analytical reagent grade.

2.2. Preparation of N-4472/VC evaporate (molar ratio: 1/5)

Four gram of N-4472 and 6.1 g of VC were dissolved in 300 ml of ethanol. The solution was evaporated under a vacuum on water bath at 60 °C. The solid mass obtained was further dried in a vacuum drier at 70 °C for 2 h and then pulverized.

2.3. Preparation of microemulsion (A) and aqueous solution (B)

2.525 g of N-4472/VC evaporate was dissolved in 40 ml of 0.01% SDS aqueous solution. The solution was added to the melted mixture of Gelucire® 44/14 (7.0 g) and HCO-60® (3.0 g) at 70 °C and mixed well with a vortex mixer to obtain N-4472 microemulsion (A). N-4472/VC

Table 1
Composition of various specimens used for physicochemical characterization and oral absorption study

	A[a]	B[b]	C[c]	D[d]
N-4472/VC evaporate[e] (mg)	50.5	50.5	–	–
VC (mg)	–	–	30.5	–
Gelucire® 44/14 (mg)	140.0	–	140.0	140.0
HCO-60® (mg)	60.0	–	60.0	60.0
SDS (mg)	0.08	–	0.08	0.08
Distilled water or D_2O (ml)	0.8	1.0	0.8	0.8

[a] A, N-4472 microemulsion.
[b] B, N-4472/VC evaporate aqueous solution.
[c] C, microemulsion without N-4472.
[d] D, microemulsion without N-4472 and VC.
[e] VC, L-ascorbic acid, N-4472/VC(molar ratio 1/5; weight ratio 20/30.5).

aqueous solution (B) was prepared by dissolving 2.525 g of N-4472/VC evaporate in 50 ml of distilled water. For ^{1}H-NMR studies, D_2O was used as a solvent (Table 1).

2.4. ^{1}H-NMR measurement

^{1}H-NMR spectrum for each specimen was measured at 24.0 °C on a JEOL JNM-LA400 spectrometer (JEOL Ltd, Japan) operating at 399.65 MHz for proton in D_2O or CD_3OD solution. Measurement conditions were as follows: 90° pulse width, 6.25 μs; relaxation delay, 2.9007 s; scan, 16 times. TMS or DSS was used as an internal standard.

2.5. Solubility determination in the various pH solutions

To determine the pH profiles of N-4472 solubility in the microemulsion (A, pH 3.0) or aqueous solution (B, pH 3.0), the pH of each specimen was adjusted to pH 1.0, 2.0, 4.0, 5.0, 6.0 and 7.0 with 0.5 M diluted HCl or 2.0 M NaOH aqueous solution. The microemulsions and aqueous solutions of various pHs were shaken for 30 min at 150 strokes/min in a water bath thermostatted at 37 °C and were filtered through a membrane filter (0.45 μm, GL Sciences Inc., Japan). Further, to evaluate the stability of the microemulsions at pHs of 2.0–7.0, the microemulsions were shaken for 1.0, 2.0 and 4.0 h at 37 °C and were filtered by the same method as described above. The concentrations of N-4472 in the solution were determined by HPLC (LC-6A, Shimadzu Co., Japan). The mobile phase (acetonitrile/distilled water/phosphoric acid (100:100:1, v/v/v)) was delivered at a flow rate of 1.0 ml/min through a L-Column® ODS (4.6 mm I.D. × 15 cm: CERI, Japan) at 40 °C. The detection wavelength was 274 nm.

2.6. Measurement of particle size distribution

The volumetric particle size distribution for N-4472 microemulsion (A) at pHs of 2.0–7.0 was measured without diluting at 25 °C by dynamic light scattering on a Microtrac® UPA (UPA 150, Nikkiso Co., Ltd, Japan).

2.7. Absorption studies

Twelve male Sprague–Dawley rats weighing 280–350 g were divided into four groups. The three rats of the first and second groups were fed with free access to the diet and the three rats of the third and fourth groups were fasted overnight before the experiment but had free access to water. N-4472 microemulsion (A) was administered orally to the three rats of the first and third groups by oral sonde in equivalent dose of 270 mg/kg of N-4472. N-4472/VC aqueous solution (B) was administered to the three rats of the second and fourth groups in the same manner. Blood samples were collected in volumes of 0.5 ml from each rat at 0.5, 0.75, 1.0, 2.0 4.0 and 6.0 h after oral administration. Plasma was separated by centrifu-

gation and stored at −20 °C until analysis. The concentration of N-4472 in plasma was determined by HPLC. Three hundred microliter of distilled water were added to 200 μl of plasma in a test tube and mixed well. N-4472 was extracted from the mixture using 6 ml of ethylacetate. After centrifugation for 10 min at 3000 × g, 4.0 ml of the organic layer was transferred into a test tube and evaporated. The residue was reconstituted with 100 μl of the mobile phase before injection into HPLC. HPLC analysis was carried out using Nanospace® system, pump 2001, UV–VIS spectrophotometric detector 2002, autosampler 2023 and column oven 2004 (Shiseido Co. Ltd, Japan). The mobile phase (acetonitrile/0.2 M ammonium acetate buffer with pH 3.7 (7:8, v/v)) was delivered at a flow rate of 300 μl/min through a J,sphere® ODS H-80 (2.0 mm I.D. × 15 cm: YMC Co. Ltd, Japan) at 40 °C. The detection wavelength was 275 nm. The area under the plasma concentration–time curve (AUC) from 0 to 6.0 h was calculated using the trapezoidal rule. The maximal plasma concentration of N-4472 (C_{max}) and the time to reach maximum plasma concentration (T_{max}) were determined as an average value calculated from actual plasma concentration profiles.

3. Results and discussion

3.1. Molecular state of N-4472/VC complex in microemulsion droplets

We reported that the N-4472/VC evaporate (molar ratio: 1/5) provided a surface-active complex which associated spontaneously to form micelle in aqueous solution (Itoh et al., submitted for publication). It was also reported that N-4472 microemulsion (A, Table 1) was stabilized by adding Gelucire® 44/14, HCO-60® and SDS (Itoh et al., 2002a). The microemulsion consisted of fine droplets with a mean size of 17.7 ± 4.8 nm.

Molecular assembling modes in microemulsion droplets were assumed to be complicated because the system consisted of numerous components such as drug, oils and surfactants. ^{1}H-NMR spectroscopic study for the microemulsion containing the N-4472/VC complex was performed in order to elucidate the molecular states of the N-4472/VC complex in microemulsion droplets by analyzing the linewidths and chemical shifts of NMR signals derived from N-4472 molecules. We focused on the NMR signals of aromatic protons, which were observed separately from those of the surfactants. Fig. 1 shows the ^{1}H-NMR spectra of the N-4472 microemulsion (A) and the N-4472/VC evaporate aqueous solution (B) prepared by using D_2O. The signals derived from the aromatic ring protons of N-4472 were observed to be around 7.6 ppm (aromatic ring III), 7.0 ppm (aromatic ring II), 6.8 ppm (aromatic ring I) and 6.6 ppm (aromatic ring I). The halfwidths of the aromatic ring protons (aromatic ring II) of N-4472 microemulsion (A) and aqueous solution (B) were estimated as 6.0 and 64.5 Hz, respectively. The halfwidth observed in N-4472 microemulsion (A) was comparable to that of an N-4472/VC evaporate methanol solution (1.5 Hz), whereas N-4472/VC evaporate aqueous solution (B) showed significantly broad peak. In addition, a similar tendency was observed in the linewidths of the other aromatic ring protons (aromatic rings I and III). Hu et al. determined the ^{1}H-NMR spectra of a pradimicin derivative in a D_2O and in a DMSO-d_6 solution, and compared the linewidths of their signals. The linewidth of the signal obtained from the D_2O solution was broader than that obtained from the DMSO-d_6 solution. They reported that molecules of the amphiphilic pradimicin derivative self-associated to form aggregates in the D_2O solution, but they did not form aggregates in the DMSO-d_6 solution (Hu et al., 1999). Considering from the observed differences in the linewidths between microemulsion (A) and aqueous solution (B), two modes of molecular state could be proposed for the N-4472/VC complex: a molecularly dispersed state in microemulsion (A) resulting in narrow NMR signals and a self-associated state in aqueous solution (B) resulting in broad NMR signals. The chemical shift of the aromatic rings proton of N-4472 in microemulsion (A) was different from that in aqueous solution (B). These results could be attributed to the difference in the environment around N-4472 between microemulsion (A) and aqueous solution (B).

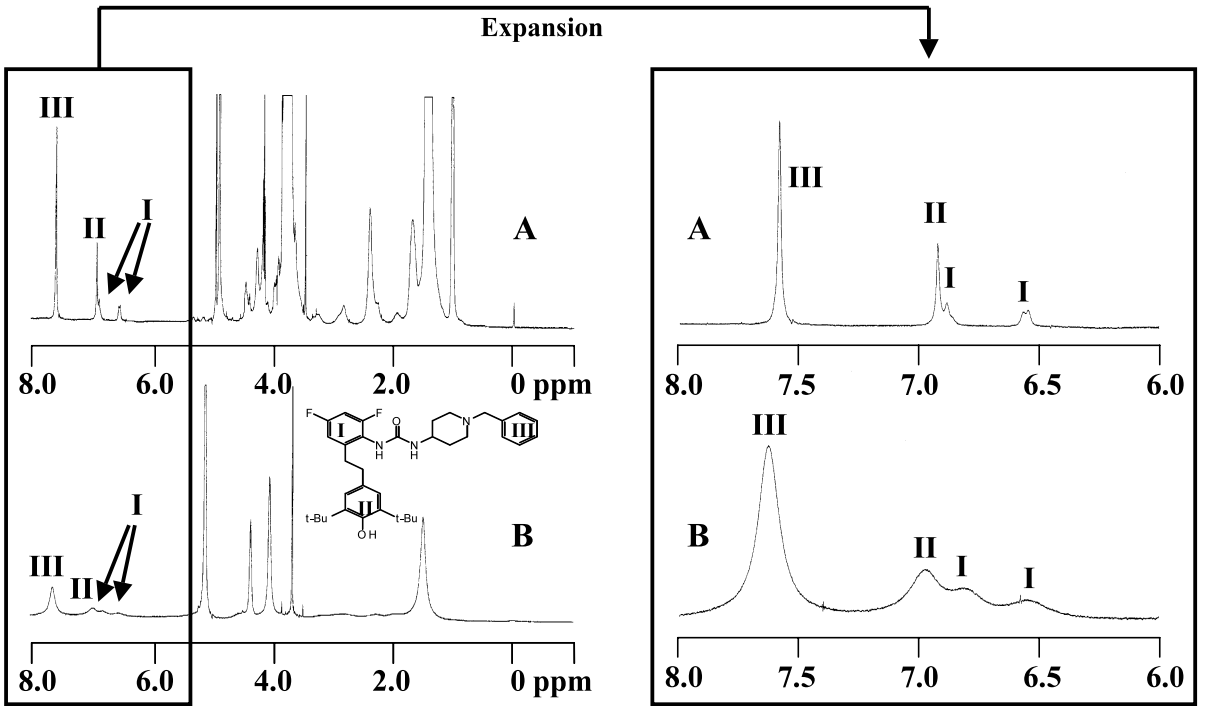

Fig. 1. Change in linewidths and chemical shifts of ^{1}H-NMR signals of aromatic ring of N-4472. (A) N-4472 microemulsion; (B) N-4472/VC evaporate aqueous solution.

In order to obtain information about the molecular state of the surfactant in the microemulsion (A), we determined the ^{1}H-NMR spectra of two other microemulsions that did not contain the N-4472/VC complex as control samples (C and D, Table 1). Fig. 2 shows the ^{1}H-NMR spectra of the microemulsions (C and D) prepared by using D_2O. The characteristic signals from surfactants (Gelucire® 44/14 and HCO-60®) observed in each spectrum were attributed to CH_3 protons (0.9–1.0 ppm) and CH_2 protons (1.3–1.4 ppm) of the fatty acid moieties and OCH_2CH_2 protons (3.6–3.8 ppm) of polyoxyethylene moieties. No significant difference was observed between microemulsion (C) and (D) in NMR chemical shifts of surfactants, while each signal of fatty acid and polyoxyethylene moieties of surfactant in microemulsion (A) shifted downfield by approximately 0.2 ppm compared with the signals of other microemulsions (C and D). These results indicated that the microenvironment around the surfactant molecules in microemulsion (A) was affected by the presence of N-4472/VC complexes. Therefore, the variation in the chemical shift of both N-4472/VC

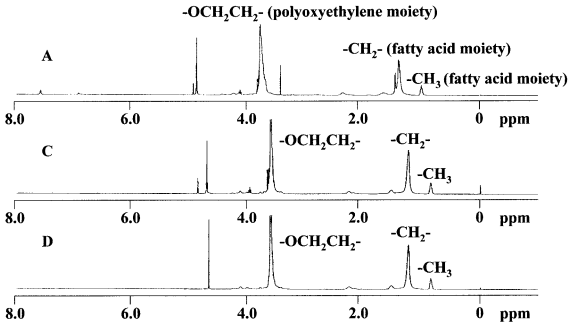

Fig. 2. Effect of corporation of N-4472/VC complex molecules on change in ^{1}H-NMR chemical shifts of polyoxyethylene and fatty acid moieties of surfactant molecules in microemulsion. (A) N-4472 microemulsion; (C) microemulsion without N-4472; (D) microemulsion without N-4472 and VC.

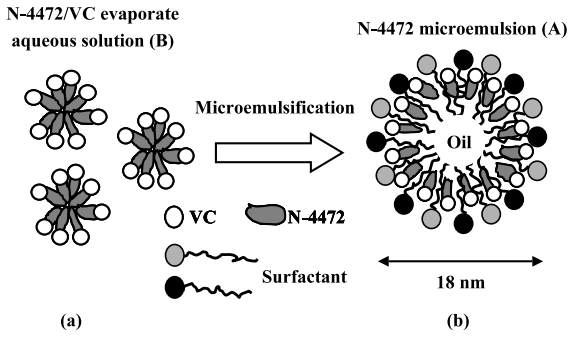

Fig. 3. Schematic representation of structural change of N-4472/VC complex molecules by microemulsification process.

complex and surfactant molecules might indicate an interaction between the N-4472/VC complex and the surfactant layer in microemulsion droplets.

Fig. 3 shows a schematic representation of the structure change of the N-4472/VC complex in microemulsion (A) and in aqueous solution (B). As shown in Fig. 3(a), the N-4472/VC complex molecules that have surface-active activity were self-associated to form aggregates in aqueous solution (B), and therefore, molecular mobility of the complexes was restricted (Itoh et al., submitted for publication). On the other hand, in Fig. 3(b), the complex molecules were molecularly incorporated into the surfactant molecules and interacted with the surfactant molecules. Disaggregation of the complex aggregates resulted in an increase in the molecular mobility of the complexes.

3.2. Effect of pH on the stability of N-4472 microemulsion droplets

The solubility of N-4472 in the microemulsion (A) and aqueous solution (B) was evaluated at different pH levels. Fig. 4 shows the pH-concentration profiles of N-4472 in microemulsion (A) and in aqueous solution (B). Most of the N-4472 fraction could not be dissolved in the aqueous solution except at pH 3.0. In the aqueous solution (B), the change in the ionic state of N-4472 and VC caused the dissociation of N-4472/VC complex, followed by the liberation of N-4472. On the other hand, when the pH of microemulsion (A) was adjusted ranging from 2.0 to 7.0, no liberation of N-4472 was observed. At pH 1.0, however, 17% of N-4472 was liberated. The instability of microemulsion at pH 1.0 was considered to result from hydrolysis of HCO-60® (Murata et al., 1990).

Further, the stability of the microemulsions at pH of 2.0–7.0 was evaluated from the N-4472 concentration in the microemulsion droplets and the mean particle size. When shaking the microemulsions at various pHs for 4.0 h at 37 °C, the change of the N-4472 concentration and the particle size are shown in Fig. 5 and Fig. 6, respectively. No liberation of N-4472 was observed for 4.0 h in each microemulsion. The mean size of microemulsion at various pHs were found between 14 and 22 nm even after incubation

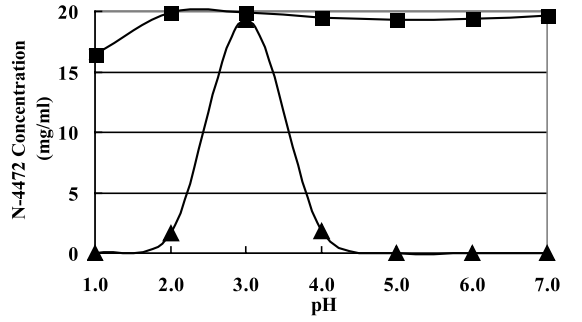

Fig. 4. Effect of microemulsification on pH-concentration profiles of N-4472/VC complex. *Key:* (A) N-4472 microemulsion (–■–); (B) N-4472/VC evaporate aqueous solution (–▲–).

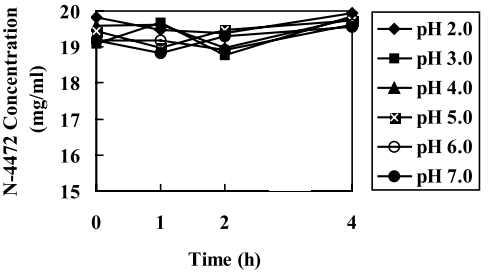

Fig. 5. Change in N-4472 concentration in the microemulsion droplets while shaking the microemulsions at pHs of 2.0–7.0 for 4.0 h at 37 °C.

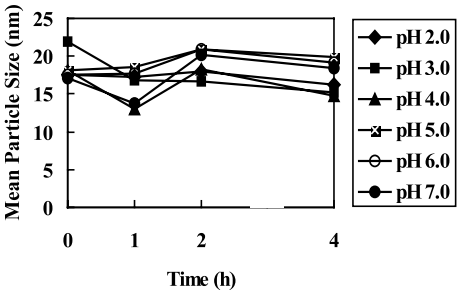

Fig. 6. Change in the mean particle size of the microemulsion while shaking the microemulsions at pHs of 2.0–7.0 for 4.0 h at 37 °C.

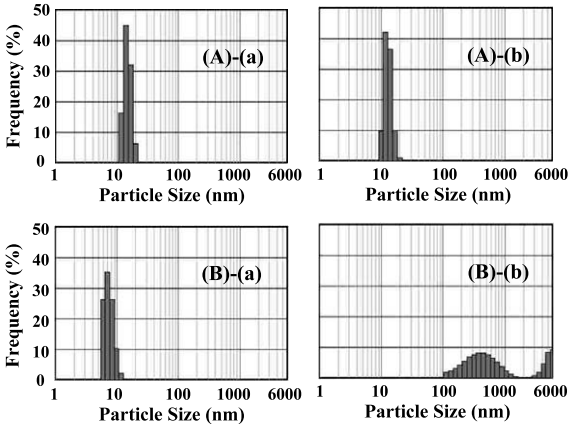

Fig. 7. Change in the particle size distributions by dispersing N-4472 microemulsion (A) and N-4472/VC evaporate aqueous solution (B) into JP XIV second fluid (pH 6.8) containing 0.02 M GCDCA-Na. *Key:* (A) (a) N-4472 microemulsion (A); (b) after dispersing microemulsion (A) into test fluid; (B) (a) N-4472/VC evaporate aqueous solution (B); (b) after dispersing aqueous solution (B) into test fluid.

for 4 h. Chen et al. reported that surfactants existing on the emulsion interface functioned as a shield to protect internal phase against changes in pH (Chen et al., 2000). The present results also indicated that the complexes were solubilized stably by being incorporated in the microemulsion droplets.

In order to presume the stability of N-4472 microemulsion (A) or aqueous solution (B) in the gastrointestinal tract after administration, the change in the particle size distribution was examined by dispersing 1.0 ml of each solution into 4.0 ml of the JP XIV second fluid (pH 6.8) containing 0.02 M GCDCA-Na. The results are shown in Fig. 7. The aqueous solution (B) contained fine particles with a mean size of approximately 9 nm before dispersing. After dispersing into test fluid the formation of large particles with a wide distribution in the range of 0.1–6.0 μm were observed. On the other hand, with regard to the microemulsion (A), no difference of size distribution was observed before and after dispersing. It was found that a microemulsion with a narrow distribution and a mean size of approximately 18 nm stably remained even in the JP XIV second fluid (pH 6.8) containing 0.02 M GCDCA-Na. Consequently, we might presume that N-4472 microemulsion (A) was stable in the gastrointestinal tract.

3.3. Oral absorption of N-4472 microemulsion

In order to investigate the oral absorption of N-4472 from the microemulsion, N-4472 microemulsion (A) was orally administered to fasted or non-fasted rats, and the concentrations of N-4472 in the plasma were determined up to 6 h after oral administration. The N-4472/VC evaporate aqueous solution (B) was also evaluated as a control sample. The plasma concentration versus time profiles are shown in Fig. 8. The pharmacokinetic parameters are shown in Table 2. Administration of microemulsion (A) to fasted rats provided great C_{max} and $AUC_{0-6\ h}$ values, which were 1.8- and 2.4-fold greater than those observed in aqueous

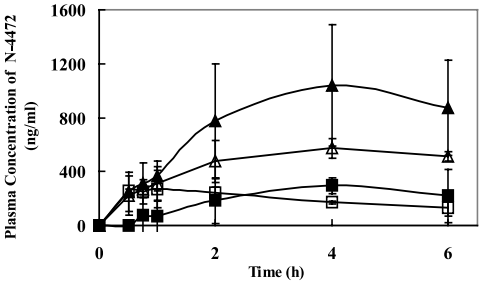

Fig. 8. Mean plasma concentration versus time profiles of N-4472 after oral administration to rats (mean ± SD, $n = 3$). *Key:* (A) N-4472 microemulsion (–△–), fasted rat; (B) N-4472/VC evaporate aqueous solution (–□–), fasted rat; (A) N-4472 microemulsion (–▲–), non-fasted rat; (B) N-4472/VC evaporate aqueous solution (–■–), non-fasted rat.

Table 2
Pharmacokinetic parameters after oral administration of N-4472 (270 mg/kg) to fasted and non-fasted rats (mean ± SD, $n = 3$)

Parameters	Fasted condition		Non-fasted condition	
	A[a]	B[b]	A[a]	B[b]
T_{max} (h)	3.3 ± 1.2	0.7 ± 0.3	4.0 ± 0.0	5.3 ± 1.2
C_{max} (ng/ml)	574 ± 75[c]	318 ± 96	1041 ± 451	308 ± 57
$AUC_{0\rightarrow 6\ h}$ (ng h/ml)	2718 ± 536[c]	1118 ± 215	4520 ± 1983[c]	1148 ± 499

[a] A, N-4472 microemulsion.
[b] B, N-4472/VC evaporate aqueous solution.
[c] $P < 0.05$, significantly different from B under same condition.

solution (B), respectively. When microemulsion (A) was administrated to non-fasted rats the C_{max} and $AUC_{0-6\ h}$ of N-4472 were 3.4- and 3.9-fold greater than those of aqueous solution (B), respectively. These results suggested that N-4472 microemulsion (A) formulation demonstrated improved absorption of N-4472 to both fasted and non-fasted rats. The enhanced absorption may be explained in terms of: (1) the huge specific surface area of the microemulsion droplets (mean droplet size: approximately 18 nm), acting advantageously in contact with the gastrointestinal tract; (2) improved release of N-4472 due to the N-4472/VC complex existing in a molecularly dispersed state in the microemulsion droplets; and (3) stability of the microemulsion in the gastrointestinal tract. With regard to feeding effect on microemulsion (A) absorption, the C_{max} and $AUC_{0-6\ h}$ in non-fasted rats were 1.8- and 1.7-fold greater than those of fasted rats, respectively. These results might be attributed to the prolonged gastrointestinal residence of N-4472 microemulsion with the decrease of the gastrointestinal emptying rate by feeding.

In conclusion, we prepared the stable microemulsion at the pH range from 2.0 to 7.0, consisting of fine droplets with a mean size of approximately 18 nm with a narrow distribution. By microemulsification, the N-4472/VC complex was molecularly incorporated into the surfactant molecular assembly in the microemulsion droplets. Since N-4472 microemulsion significantly improved the oral absorption, irrespective of feeding addition, it was clarified that microemulsion droplet would be useful as a drug carrier.

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