Research Article

¹⁹F Nuclear Magnetic Resonance Spectrometric Determination of the Partition Coefficients of Flutamide and Nilutamide (Antiprostate Cancer Drugs) in a Lipid Nano-Emulsion and Prediction of Its Encapsulation Efficiency for the Drugs

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Abstract. To design a useful lipid drug carrier having a high encapsulation efficiency (*EE*%) for the antiprostate cancer drugs flutamide (FT) and nilutamide (NT), a lipid nano-emulsion (LNE) was prepared with soybean oil (SO), phosphatidylcholine (PC), and sodium palmitate, and the partition coefficients (K_p s) of the drugs for the LNE were determined by ¹⁹F nuclear magnetic resonance (NMR) spectrometry. The ¹⁹F NMR signal of the trifluoromethyl group of both drugs showed a downfield shift from an internal standard (trifluoroethanol) and broadening according to the increase in the lipid concentration due to their interaction with LNE particles. The difference in the chemical shift ($\Delta\delta$) of each drug caused by the addition of LNE was measured under different amounts of LNE, and the K_p values were calculated from the $\Delta\delta$ values. The results showed that FT has higher lipophilicity than NT. The total lipid concentration (SO + PC) required to encapsulate each drug into LNE with an *EE*% of more than 95% was calculated from the K_p values as 93.3 and 189.9 mmol/L for FT and NT, respectively. For an LNE prepared with the total lipid concentration of 215 mmol/L, the predicted *EE*% values were 98 and 96% for FT and NT, respectively, while the experimental *EE*% values determined by a centrifugation method were approximately 99% for both drugs. Thus, the ¹⁹F NMR spectrometric method is a useful technique to obtain the K_p values of fluorinated drugs and thereby predict the theoretical lipid concentrations and prepare LNEs with high *EE*% values.

KEY WORDS: ¹⁹F NMR spectrometry; antiprostate cancer drug; encapsulation efficiency; lipid nano-emulsion; partition coefficient.

INTRODUCTION

Flutamide (FT) and nilutamide (NT), as shown in Fig. 1, are non-steroidal antiandrogen drugs used to treat prostate cancer. Administrations of FT and NT frequently cause serious adverse reactions such as gynecomastia and hepatotoxicity due to the distribution of these drugs throughout the whole body (1). The drug carriers in drug delivery systems (DDS) have been developed as a potentially effective tool for improving the therapeutic index of drugs by increasing their efficacy or reducing their toxicity *in vivo* via the accumulation and controlled release of drugs at their target sites. Therefore, a variety of drug carriers for FT have been investigated, e.g., liposome (2), hydrogel (3), cyclodextrins (4), casein nanoparticles (5), chitosan-dextran sulfate nanoparticles (6), and poly(lactide-co-glycolide) nanoparticles (7). To our knowledge, however, there has been no report on the preparation of drug carriers for NT.

Commercially available lipid emulsions (LEs), which are composed of vegetable oil and phosphatidylcholine (PC), are widely used as not only parenteral nutrition but also drug carriers. For cancer chemotherapy, drug carriers are required to have a particle size of less than 100 nm (8, 9) because they can then accumulate passively and selectively in tumor tissues owing to leaky tumor vasculature by the enhanced permeation and retention (EPR) effects (10). However, the commercial LEs generally have a droplet size ranging from 200 to 300 nm, which is too large to pass through the discontinuous capillary endothelium of tumors (11). Therefore, we previously developed a lipid nano-emulsion (LNE) prepared from a lipid mixture of soybean oil (SO), PC, sodium palmitate (PANa), and sucrose palmitate (12). Because the mean droplet size of this and our subsequently developed LNEs are approximately 50 nm, they can be used as DDS carriers for cancer therapy (13, 14). In addition, the LNEs have a major advantage over other DDS carriers in that FT is insoluble in water but very soluble in SO, i.e., its solubilities in water and SO are 9.45 mg/L and 7.6 mg/mL, respectively (15). Therefore, the LNEs can encapsulate large amounts of FT and are thus expected to be useful carriers for non-steroidal antiandrogen drugs.

The *n*-octanol/water partition coefficient of drugs (log P_{oct}) is widely used as a hydrophobic index to evaluate the drug encapsulation ability of drug carriers, especially lipid

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carriers, e.g., liposomes and LEs. However, we have recently reported that there is no significant relationship between the partition coefficients in liposome/water and *n*-octanol/water systems (16), suggesting that the lipophilic nature is not completely consistent with the hydrophobic one. Although the use of a partition coefficient of drugs between drug carriers and water is thus desirable, such studies have not been reported previously.

¹⁹F nuclear magnetic resonance (NMR) spectroscopy is a powerful tool for investigating the interaction between compounds containing ¹⁹F atoms and biomolecules, because the ¹⁹F nucleus is not present in natural biological substances and is thus easily detectable without interfering signals (17–24). In addition, we have previously reported that the partition coefficients (*K_p*s) of some fluorinated psychotropic drugs between PC small unilamellar vesicles and water can be successfully determined without disturbing the equilibrium condition by using the ¹⁹F NMR method (25). As shown in Fig. 1, since FT and NT have a trifluoromethyl (CF₃–) group in their chemical structures, we considered that the ¹⁹F NMR method could be expanded for the determination of their *K_p* values between LNE particles and water.

In this study, we prepared LNEs containing either FT (FT-LNE) or NT (NT-LNE) with the same lipid composition as previously reported (12) and determined the *K_p* values of both drugs by an ¹⁹F NMR spectrometric method. Using the obtained *K_p* values, we then calculated the concentration of lipids required to prepare an LNE with a lipid phase containing more than 95% of the total amount of FT or NT. Based on the obtained lipid concentration, we further prepared FT- and NT-LNEs and examined their ability to encapsulate both drugs and their other characteristics. To assess the drug encapsulation ability of the LNE, we calculated the encapsulation efficiency (*EE*%) as follows:

$$EE\% = \frac{\text{The amount of drug in lipid phase in unit volume of LNE}}{\text{The total amount of drug in unit volume of LNE}} \times 100. \quad (1)$$

MATERIALS AND METHODS

Calculation of Molar Partition Coefficients

The molar partition coefficient (*K_p*) of a drug between LNE and water was defined as follows (26, 27):

$$K_p = \frac{([D_L]/[D_T])/[L]}{([D_W]/[D_T])/[W]} \quad (2)$$

where *[D_L]* and *[D_W]* represent the molar concentrations of drug in LNE and water, respectively, and *[D_T]* = *[D_L]* + *[D_W]*, and *[L]* and *[W]* are the molar concentrations of lipids, e.g., triglyceride (TG) of SO + PC in LNE, and water (55.4 mol/L at 21°C), respectively.

Since the magnetic environment of the drug partitioned in LNE is different from that of the free drug in the water phase, the chemical shift of the ¹⁹F signal of

Fig. 1. Chemical structures of flutamide and nilutamide

the partitioned drug will also be different from that of the free drug in water. We therefore express the ¹⁹F chemical shift of the drug in the lipid phase as δ_L and that in water as δ_W . If the exchange rate of the drug between the lipid and water phases is fast enough at the time scale of ¹⁹F NMR, the observed chemical shift (δ_O) of the ¹⁹F signal can be expressed as follows:

$$\delta_O = ([D_L]/[D_T])\delta_L + ([D_W]/[D_T])\delta_W.$$

A new variable, $\Delta\delta$, is introduced to represent the difference between δ_O and δ_W as follows:

$$\Delta\delta = \delta_O - \delta_W = ([D_L]/[D_T])(\delta_L - \delta_W).$$

Because the value of ($\delta_L - \delta_W$) is constant, $\Delta\delta$, the chemical shift difference of the drug before and after the addition of LNE, is proportional to the fraction of the drug in the lipid phase, ($[D_L]/[D_T]$). Thus, ($[D_L]/[D_T]$) can be shown as follows:

$$([D_L]/[D_T]) = \Delta\delta / \Delta\delta_{\max}, \quad (3)$$

where $\Delta\delta_{\max} = \delta_L - \delta_W$.

Using Eqs. (2) and (3), it can be rewritten as follows:

$$\Delta\delta = \frac{K_p \Delta\delta_{\max} [L]}{K_p [L] + [W]}. \quad (4)$$

The values of *K_p* and $\Delta\delta_{\max}$ can be calculated from the experimental values of *[L]* and $\Delta\delta$ by applying a non-linear least-square calculation to Eq. (4). This calculation was performed using a Visual Basic program written in our laboratory (25).

Fraction of FT and NT in LNE Particles

If the *K_p* value is obtained, the fraction (%) of drug in the lipid phase of LNE at a lipid concentration *[L]* is calculated from Eqs. (3) and (4) as follows:

$$F\% = \frac{[D_L]}{[D_T]} \times 100 = \frac{\Delta\delta}{\Delta\delta_{\max}} \times 100 = \frac{K_p [L]}{[W] + K_p [W]} \times 100. \quad (5)$$

From the definition of *EE*% in Eq. (1), it is clear that *F*% = *EE*%.

We can thus predict the lipid concentration, *[L]*, required to obtain a high *EE*% value of LNE for these drugs.

Materials

FT, NT, and PANa were purchased from Sigma-Aldrich (St. Louis, MO). L- α -PC (egg yolk) of 99% purity was supplied as a 2% (w/v) chloroform solution from Avanti Polar-Lipids Inc. (Alabaster, AL) and used without further purification after the purity of PC was confirmed by thin-layer chromatography that showed a single spot by spraying 10% sulfuric acid. SO and glycerol were purchased from Kanto Chemical Co. (Tokyo, Japan). The buffer used was 10 mmol/L 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes buffer, pH 7.4, 21°C) containing 10% deuterium oxide (D₂O) for a deuterium lock signal. Other reagents were of analytical reagent grade.

Preparation of LNE

Each of 0.03 g FT or NT, 4.5 g SO, 1.08 g PC, and 0.108 g PANa was added to a glass container. Then, deionized distilled water containing glycerol (2.2 (w/v) %) was added to bring the total volume to 30 mL so that the final drug concentration was 1 mg/mL. The mixture was vigorously stirred with a pencil mixer. The FT- and NT-LNEs were then prepared by sonication as described in detail elsewhere (12). An LNE containing neither FT nor NT was also prepared in the same manner with the same lipid composition as a control.

Characterization of LNE

The LNEs were further diluted with Hepes buffer to 1:1000 for droplet size measurement and to 1:10,000 for zeta potential measurement. The diameter and size distributions of the LNE particles were determined by a dynamic light scattering (DLS) method using a Nicomp Model 380 particle sizer (Particle Sizing Systems Inc., Santa Barbara, CA). The LNE droplet size was reported as the volume weighted distribution. The zeta potential value was measured by a Zeecom ZC-3000 analyzer (Microtec Co., Ltd., Chiba, Japan) based on the principle of electrophoresis.

Determination of Lipid Concentration

The molar quantity of SO was assumed to be equivalent to that of triacylglycerol (TG; average molecular weight of 900) in SO. The amount of TG in SO was determined by the enzyme-reaction method using glycerophosphate oxidase (28). The exact PC concentration in the LNE was calculated from phosphate analysis according to the phosphovanadomolybdate method (29).

Measurements of ¹⁹F NMR Spectra

To each of several 2-mL volumetric flasks containing a suitable amount of FT or NT stock solution, a suitable aliquot

Fig. 2. ¹⁹F NMR spectra of 0.2 mmol/L flutamide in 10 mmol/L Hepes buffer (pH 7.40, 21°C) containing various amounts of LNE. The internal standard (0 ppm) is trifluoroethanol. Lipid concentration (mmol/L): (1) 0.00, (2) 1.81, (3) 4.53, (4) 6.34, (5) 9.06, (6) 18.12, and (7) 27.18

of the LNE was added, and 200 μ L of 2 mmol/L TFE stock solution was then added as an internal standard (200 μ mol/L). Finally, the buffer was further added to volume.

After shaking each flask for a short time, 1 mL of the sample solution was transferred to a 5-mm-diameter NMR tube. ¹⁹F NMR spectra were measured by using a UNITY INOVA spectrometer (Agilent Technologies Inc., Santa Clara, CA) operating at 376.21 MHz without proton decoupling. The set parameters were a 4.4- μ s pulse width (45° for the flip angle) and an acquisition time of 0.5 s. The probe temperature was 21 $\pm$ 1°C. The number of free induction decay (FID) accumulations to improve the signal to noise (S/N) ratio was 1000.

Encapsulation Efficiency of LNE for FT and NT

Five hundred microliters of FT- or NT-LNE was introduced into the centrifugation tube with an ultrafiltration membrane having a molecular weight cut-off of 10 K (Amicon® Ultra-4; Millipore Co., Billerico, MA) and then centrifuged for 20 min at 2000 $\times$ g. After the centrifugation, FT or NT in the filtrate in the bottom of the centrifugation tube, i.e., the FT or NT in the bulk water, was analyzed by HPLC. The EE% of both drugs was calculated from Eq. (6) as shown below (14):

$$EE\% = \frac{\text{Total amount of drug added for the preparation (mg)} - \text{Amount of drug in the water phase (mg)}}{\text{Total amount of drug added for the preparation (mg)}} \times 100. \quad (6)$$

Table I. The K_p and $\Delta\delta_{\max}$ Values of FT and NT in LNE at Several Drug Concentrations

Drug	$K_p \times 10^{-3}$			$\Delta\delta_{\max} \times 10$ (ppm)		
	Drug concentration (mmol/L)			Drug concentration (mmol/L)		
	0.1	0.2	0.3	0.1	0.2	0.3
FT	29.0 ± 0.3	18.0 ± 0.2	11.3 ± 0.6	1.85 ± 0.01	1.87 ± 0.14	1.79 ± 0.03
NT	12.7 ± 0.3	9.0 ± 0.6	5.6 ± 0.2	1.53 ± 0.01	1.39 ± 0.04	1.36 ± 0.01

Each value is expressed as the mean ± standard deviation ($n = 3$)

FT flutamide, NT nilutamide

HPLC Measurement of the FT and NT Concentrations

The quantitative determination of FT and NT was performed by the HPLC method on an LC-10AT (Shimadzu, Kyoto, Japan) using a COSMOSIL 5C18-MS-II column (4.6 × 150 mm, 5 μm; Nacalai Tesque, Kyoto, Japan) with a mobile phase containing a mixture of acetonitrile and water (9:1, v/v) at a flow rate of 0.5 mL/min. A sample was dissolved in ethanol to a suitable drug concentration before injection. The sample injection volume was 20 μL, and the detection of both drugs was performed using a UV detector at a wavelength of 295 nm.

RESULTS AND DISCUSSION

¹⁹F NMR Spectra

The ¹⁹F NMR spectra of 0.2 mmol/L FT in the sample solutions containing various amounts of LNE are depicted in Fig. 2 as a typical example. As shown in Fig. 2, without LNE, a single sharp signal arising from the CF₃– group of free FT was observed at 16.58 ppm, while that of free NT was observed at 16.56 ppm (data not shown). However, in the presence of LNE, FT showed a downfield shift and signal broadening according to the increase in the lipid concentration, indicating that FT partitions from the aqueous phase to the lipid phase in the sample suspension. The downfield shift of F signals can be

attributed to the difference between the bulk magnetic susceptibilities of the water and the lipid phases (30–32). In addition, FT provided a single signal at all lipid concentrations studied. This result means that the exchange rate of FT between the free and partitioned states is fast enough relative to the ¹⁹F NMR time scale. Therefore, these results demonstrated that the K_p value of FT can be theoretically calculated from Eq. (4) using the $\Delta\delta$ value. Also, NT showed similar results and the K_p value of NT could be obtained in the same way.

Calculated K_p Values

The $\Delta\delta$ values for FT and NT were obtained from the ¹⁹F NMR spectra measured in the presence of various amounts of LNE, and then the obtained $\Delta\delta$ values were used to calculate the K_p and $\Delta\delta_{\max}$ values. The K_p and $\Delta\delta_{\max}$ values of FT and NT at various drug concentrations are listed in Table I; their relative standard deviations were below 8%, indicating the good precision of the ¹⁹F NMR spectrometric method. In addition, FT and NT, respectively, show similar $\Delta\delta_{\max}$ values for the experimentally employed three different drug concentrations. The result confirms the validity of applying partition theory in this study.

FT showed an approximately twofold higher K_p value than NT at the same drug concentration. Since the reported log P_{oct} values are 2.6 for FT (15) and 2.2–2.3 for NT (33,34), respectively, FT has not only higher hydrophobicity but also

Fig. 3. Fraction (%) of FT (a) and NT (b) in LNE particles at the drug concentrations of 0.1 (black circle), 0.2 (black triangle), and 0.3 (black square) mmol/L as a function of lipid concentration. The solid lines represent the theoretical curves calculated from Eq. (5) using the experimental values

Table II. Comparison of Particle Size, Zeta Potential, and *EE*% of LNE, FT-LNE, and NT-LNE

LNEs	Particle size ^a	Zeta potential ^a	<i>EE</i> %	
	(nm)	(mV)	Predicted	Measured ^b
LNE	42 ± 5	-22.5 ± 2.3	–	–
FT-LNE	48 ± 4	-35.6 ± 3.0	98	>99
NT-LNE	54 ± 6	-26.7 ± 1.8	96	>99

LNE lipid nano-emulsion, FT-LNE flutamide lipid nano-emulsion, NT-LNE nilutamide lipid nano-emulsion, *EE*% encapsulation efficiency. The drug and total lipid concentrations are 0.3 and 215 mmol/L, respectively

^a Each value is expressed as the mean ± standard deviation ($n = 3$)

^b The determinations were carried out in triplicate

higher lipophilicity than NT. Therefore, if these drugs are encapsulated in LNE particles, FT should show a higher *EE*% value than NT.

On the other hand, as seen in Table I, the K_p values of FT and NT showed a decreasing tendency that was inversely dependent on the drug concentration. It is known that the K_p values of certain amphiphilic drugs have a concentration dependence, due to molecular association or formation of micelles (35, 36). The decrease in the K_p values of FT and NT may also have been due to the formation of a molecular association caused by the intermolecular hydrogen bond at their amide groups, which depends on the increase in the number of drug molecules in the sample solutions.

Fraction% of FT and NT in LNE Particles

In Fig. 3, the *F*% profiles of FT (a) and NT (b) partitioned into the LNE particles are shown as a plot of the $\Delta\delta/\Delta\delta_{\max}$ values *versus* the lipid concentration. Solid lines represent the theoretical curves calculated from Eq. (5) using the obtained K_p and $\Delta\delta_{\max}$ values. The experimental values for both drugs show a good fit with the calculated curves, indicating the validity of the obtained K_p values.

Using these K_p values, the *F*% values of FT and NT can be calculated for a given lipid concentration. Inversely, we can calculate the lipid concentration required to obtain a given *F*% value. For example, by the calculation of the *F*% values with the lowest K_p values obtained at the drug concentration of 0.3 mmol/L, it was shown that more than 95% of both drugs can be theoretically encapsulated into LNE particles at the total lipid concentrations (the sum of SO and PC) of 93.3 and 189.9 mmol/L for FT and NT, respectively.

Characterization of FT- and NT-LNEs

To determine whether an LNE with an *EE*% value higher than 95% for FT and NT can be obtained, FT- and NT-LNEs with a total lipid concentration of 215 mmol/L were prepared. Their characteristic features were examined and are shown in Table II. There was no significant difference in the droplet size between LNEs without and with drugs. However, the zeta potentials of FT-LNE and NT-LNE were different; the zeta potential of FT-LNE negatively increased to -35.6 mV compared to the value of -22.5 mV for LNE, while NT-LNE had a zeta potential of -26.7 mV which was slightly negatively higher than LNE. The negative value of LNE was mainly due to the

carboxyl (COO^-) group of PANa in LNE particles. Since the quantity of PANa was the same in all of the LNE preparations, the change of zeta potential values was derived from the drugs encapsulated in the LNE particles. Previous studies have reported pK_a values of 4.58 (37) or 4.8 (38) for FT, and thus FT exists in a deprotonated monoanionic form at pH 7.4 (38). Due to the negatively higher zeta potential value of FT-LNE, most of FT can be solubilized as a neutral form in the core SO phase of LNE particles, while a small amount of the deprotonated monoanionic form of FT may be present on the surface of LNE particles. On the other hand, the pK_a value of NT could not be found in the literature. However, two papers on the ionization of NT have been reported. One reported that NT exists in a neutral form at a wide pH range but exists as a weak anionic form at pH 13 (39). The other showed that NT has three ionization states at medium pH (40), although it was not elucidated what ionization states exist in the solution. Our results on the zeta potential of NT-LNE indicate that NT is almost solubilized as a neutral form in the core SO phase of LNE particles, while an extremely small amount of NT may exist in a monoanionic form on the surface of LNE particles.

As shown in Table II, the *EE*% values for 0.3 mmol/L FT and NT were calculated as 98 and 96%, respectively, from the K_p values of 11,300 and 5600 in Table I. On the other hand, the *EE*% values measured by a centrifugation method were larger than 99% for a drug concentration of 1 mg/mL, which corresponds to 3.6 and 3.2 mmol/L for FT and NT, respectively, and these values were much higher than the value of 0.3 mmol/L used in the predicted *EE*% calculation. These results indicate that FT and NT were almost completely encapsulated in the LNE particles. In addition, the results confirm that the K_p value of a drug for a drug carrier determined by the ^{19}F NMR spectrometric method can be useful to predict the *EE*% of the drug carrier for the drug.

CONCLUSION

We showed that the K_p values of FT and NT in LNE were easily and accurately determined by the ^{19}F NMR spectrometric method. In addition, we demonstrated that the measured *EE*% values for FT and NT were well coincident with the predicted *EE*% values calculated from their K_p values. Thus, the K_p values of drugs will provide useful theoretical

information in preliminary studies for the development of drug carriers with high *EE*%.

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declare that they have no competing interests.

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