การศึกษาทางโฟเรียทรานสฟอร์มอินฟราเรดสเปกโตรสโคปีเกี่ยวกับการเกิดพันธะไฮโดรเจน ระหว่างยากลุ่มเบนโซไดอะเซปีนและแผ่นเยื่อฟอสฟอลิปิด

นางสาว ศิริพร เหล่ามานะเจริญ

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย

FOURIER TRANSFORM INFRARED SPECTROSCOPIC STUDY ON HYDROGEN BONDING BETWEEN BENZODIAZEPINE DRUGS AND PHOSPHOLIPID

Miss Siriphorn Laomanacharoen

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

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ศิริพร เหล่ามานะเจริญ : การศึกษาทางโฟเรียทรานสฟอร์มอินฟราเรดสเปกโตรสโคปีเกี่ยวกับการเกิดพันธะไฮโดรเจนระหว่างยากลุ่มเบนโซไดอะเซปีนและแผ่นเยื่อฟอสฟอลิปิด. (FOURIER TRANSFORM INFRARED SPECTROSCOPIC STUDY ON HYDROGEN BONDING BETWEEN BENZODIAZEPINE DRUGS AND PHOSPHOLIPID) อ. ที่ปรึกษา : ผ.ศ. ดร. อุษา กล้ากสิกิจ. 126 หน้า. ISBN 974-17-4306-8.

ผลของยาเบนโซไดอะเซปีน(ไดอะซีแพมและลอราซีแพม)ต่อพันธะไฮโดรเจนในฟอสฟอลิปิดถูกตรวจโดยโฟเรียทรานสฟอร์มอินฟราเรดสเปกโตรสโคปีโดยใช้รีเวอร์สไมเซลล์และลิโปโซมเป็นรูปแบบแผ่นเนื้อเยื่อในการศึกษา ในรีเวอร์สไมเซลล์ไดอะซีแพมส่งผลต่อกลุ่มเอสเอ็น-2 คาร์บอนีลซึ่งอยู่ใกล้บริเวณไม่ชอบน้ำของโมเลกุลฟอสฟอลิปิดโดยทำลายพันธะไฮโดรเจนระหว่างน้ำและกลุ่มคาร์บอนีล ในขณะที่ตำแหน่งการมีปฏิกิริยาของลอราซีแพมจะอยู่ใกล้กับบริเวณชอบน้ำของโมเลกุลในลิโปโซมไดอะซีแพมและลอราซีแพมมีปฏิกิริยาที่กลุ่มเอสเอ็น-2 คาร์บอนีลซึ่งสามารถแปลผลได้จากการเพิ่มขึ้นของอัตราส่วนระหว่างแถบยืดหดเอสเอ็น-2 คาร์บอนีลที่เป็นอิสระและที่รวมกับน้ำหลังจากการเติมยา ลอราซีแพมให้ปฏิกิริยาแรงกว่าไดอะซีแพมเนื่องจากมีความเป็นขั้วในโมเลกุลสูงกว่าทำให้อยู่ใกล้กับตำแหน่งของการเกิดปฏิกิริยาต่อกันมากกว่า การลื่นไหลของแผ่นเยื่อและขนาดของลิโปโซมมีบทบาทสำคัญต่อการเกิดปฏิกิริยาต่อกันระหว่างยาและแผ่นเยื่อฟอสฟอลิปิด เนื่องจากตำแหน่งการเกิดปฏิกิริยาต่อกันของยาต่อพันธะไฮโดรเจนในโมเลกุลฟอสฟอลิปิดแตกต่างกันในรีเวอร์สไมเซลล์และลิโปโซม ดังนั้นในการเลือกตัวอย่างฟอสฟอลิปิดที่เหมาะสมขึ้นกับวัตถุประสงค์ในการศึกษา

สถาบันวิทยบริการ
จุฬาลงกรณ์มหาวิทยาลัย

ภาควิชา.....เภสัชเคมี.....ลายมือชื่อนิสิต.....
สาขาวิชา.....เภสัชเคมี.....ลายมือชื่ออาจารย์ที่ปรึกษา.....
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SIRIPHORN LAOMANACHAROEN : FOURIER TRANSFORM INFRARED SPECTROSCOPIC STUDY ON HYDROGEN BONDING BETWEEN BENZODIAZEPINE DRUGS AND PHOSPHOLIPID. THESIS ADVISOR : ASSIST. PROF. USA GLAGASIGIJ, Ph.D. 126 pp. ISBN 974-17-4306-8.

The effects of benzodiazepine drug (diazepam and lorazepam) on the hydrogen bonding in phospholipid were investigated by Fourier transform infrared spectroscopy using reversed micelles and liposomes as studied model membrane. In reversed micelles, diazepam provided their effect on sn-2 C=O group located closely to hydrophobic region of phospholipid molecule by interruption of hydrogen bond between water and C=O groups while site of action of lorazepam might be close to hydrophilic head group. In liposomes, diazepam and lorazepam exhibited their activity at sn-2 C=O group which could be interpreted from increasing of ratio between the free and hydrated sn-2 C=O stretching band upon drug addition. Lorazepam possessed stronger activity than diazepam due to its higher polarity in molecule that provided its location closer to site of interaction. Membrane fluidity and also size of liposome played significant role in drug interaction with phospholipid membrane. Since site of drugs interaction on hydrogen bonding in phospholipid molecule was different between reversed micelles and liposomes, therefore appropriate selection of model membrane depended on purpose of study.

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จุฬาลงกรณ์มหาวิทยาลัย

Department.....Pharmaceutical Chemistry…...Student's signature…….

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CHAPTER I

INTRODUCTION

Phopholipids are the major components of biological membranes. Their chemical structures are composed of two major parts: hydrophilic head group and hydrophobic tails. The hydrophilic head group is the region that phosphate group links with organic alcohol such as choline, ethanolamine, glycerol, serine and inositol by ester bond. The hydrophobic tail is two fatty acyl chains which have various numbers of carbon atom and unsaturated bonds depending on the type of phospholipid. The two parts link each other by forming an ester bond with glycerol which is the back bone of phospholipid molecule [New, 1990]. The phospholipid molecules in biological membranes align themselves closely in planar bilayer sheets. The bilayer is composed of two monolayer sheets of phospholipid molecules with their hydrophobic tails facing each other by Van der Waal interaction and hydrophilic head group facing the water molecule by hydrogen bond. This arrangement of phospholipid molecules is the important factor for the structure and properties of the biological membranes [Blume, 1988; Boggs, 1987; Chiou, 1990; Chiou, 1992; New, 1990; Tsai, 1987; Tsai, 1990].

Because of the appropriate arrangement of phospholipid molecule and the significant role of hydrogen bond in biological membrane, there were many previous works studied about factors influencing on biological membrane and the alteration of membrane structure. As the temperature increases, the fatty

acyl chains tend to change their conformations. This leads to decrease the bilayer thickness and expand the area of hydrophobic tail [New, 1990]. The previous studies of high pressure [Mushayakarara, 1986; Reis, 1996] , alcohols [Chiou, 1992] and anesthetics [Brockerhoff, 1982; Buchet, 1985; Chiou, 1990; Tsai, 1987; Tsai, 1990; Ueda, 1994] revealed that all these factors could interrupt the hydrogen bonds between water and phospholipid molecules and then released the water molecules. Especially, there were many studies about the effect of anesthetics on phospholipid membrane. They showed that the action site was the membrane-water interface and the anesthetic action was to break the hydrogen bonds and released bound water molecules [Tsai, 1987; Ueda, 1994]. From these studies showed that the hydrogen bond not only played an important role of supporting the membrane structure but it was also the site of action on phospholipid membrane.

The studies of phospholipid membrane have been demonstrated by many methods for example differential scanning calorimetry (DSC), electron spin resonance (ESR), Raman spectroscopy, NMR spectroscopy etc. Infrared spectroscopy is another sensitive method that can assess dynamics of each part of phospholipid molecules in the bilayer membrane. It is also the nonpertubing technique that monitors molecular vibrations and operates on a very short time. Functional groups of phospholipid molecule give the apparent infrared peaks so the alteration as well as the structural and dynamical properties of phospholipid molecule can be detected by varying in peak position, intensity, band width and occurring of new peak [Blume, 1988; Reis, 1996; Tsai, 1990]. P=O of phosphate group and glycerol C=O (sn-2) group which is next to the hydrophilic head region are the main positions hydrated by water molecules. Therefore, the loss of hydrogen bonded water molecule at the

surface of phospholipid membrane due to drugs or chemical agents can be detected easily by infrared spectroscopic technique.

The benzodiazepines are the most important sedative-hypnotics. The major molecular targets of the benzodiazepines are inhibitory neurotransmitter receptors directly activated by the amino acid, gamma-aminobutyric acid (GABA). The interaction of benzodiazepines with GABA induces the enhancement in chloride ion conductance by increasing the frequency of channel opening [Hardman, 1996]. Beside the pharmacological action via GABA receptor, Kurishingal et al. (1992) showed that benzodiazepines could fluidize the phospholipid membrane by fluorescence polarization measurement, using 1,6-diphenyl-1,3,5-hexatriene (DPH) as fluorescent probe. From the chemical structure, benzodiazepines have functional groups that can form hydrogen bond with phospholipid membrane and also give the apparent infrared peaks. However, there is no previous investigation related with the hydrogen bond interaction between benzodiazepines and phospholipid membranes. Thus, it is interesting to study the effect of benzodiazepines on hydrogen bond supporting in the phospholipid membrane to examine whether it is the site of action and its correlation with therapeutic concentration of drug. Infrared spectroscopic technique was selected as a method of choice to manipulate the hydrogen bond interaction while liposome and micelle were used as a model membrane. The obtained results may be evident for further investigation in native cell membrane as a purpose of drug monitoring.

CHAPTER II

BACKGROUND

Phospholipid

Phospholipids, the main constituents of biological membranes, have two major parts of chemical structure which link with each other by glycerol backbone: hydrophilic polar head group and hydrophobic tail as illustrated in Figure 1. The former is the area that phosphate group esterifies with organic alcohol whereas the latter is a pair of acyl hydrocarbon chains. The most common phospholipids are phosphatidyl cholines (PC) known as lecithin. Phosphatidyl cholines can be derived from both natural and synthetic sources. Lecithin from natural sources is a mixture of phosphatidyl cholines, each with different chain length and varying degrees of unsaturation [New, 1990].

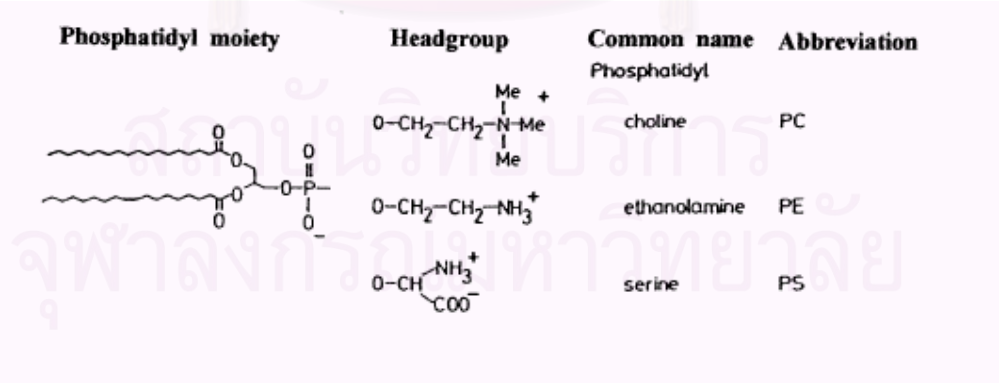

Figure 1 Some common naturally-phosphatidyl phospholipids.

Phospholipids form a bimolecular structure with two layers. The hydrophilic polar head groups will be at the interface facing the aqueous medium and the hydrophobic tails will interact to form an environment that excludes water. The phospholipid bilayers (Figure 2) are held together by Van der Waal interactions of the hydrocarbon chains and hydrogen bonds of the polar head groups with water [Blume, 1988; Boggs, 1987; Chiou, 1990; Chiou, 1992; New, 1990; Tsai, 1990; Tsai, 1987].

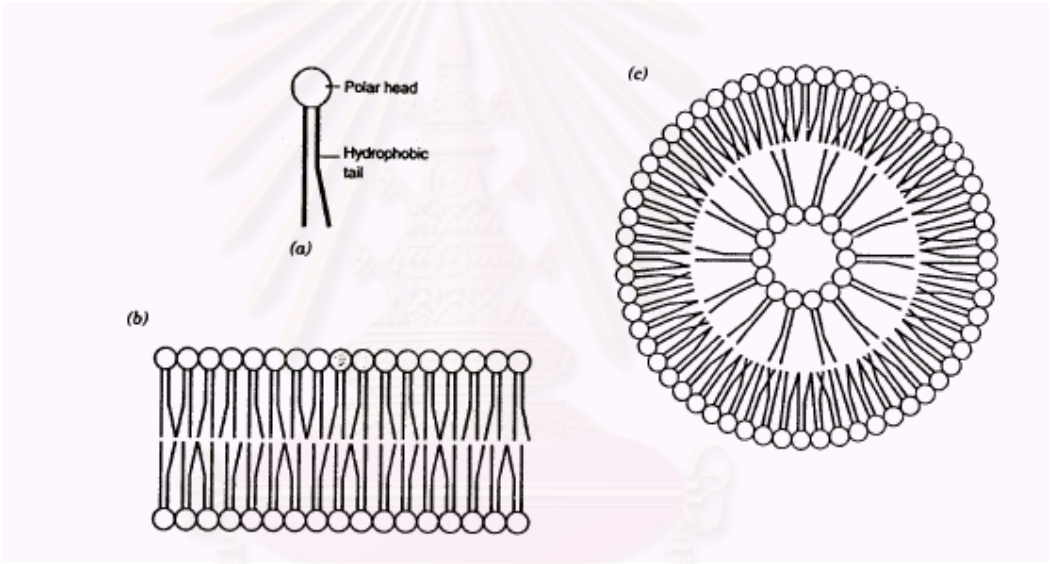

Figure 2 Representation of the phospholipids in an aqueous medium.
(a) Representation of an amphipathic phospholipid.
(b) Cross-sectional view of the structure of phospholipid bilayer.
(c) Cross-sectional of a liposome.

Figure 3 shows the stratification of biological membrane clearly demonstrating the location of hydrogen bond region (hydrogen belt) and other constituents such as proteins and cholesterol. The hydrogen bond region contains hydrogen bond acceptors (C=O of phospholipids) and hydrogen bond donors (OH of cholesterol, water). Manipulation of the composition of the hydrogen belt changes the structure of the hydrogen bond network [Brockerhoff, 1982].

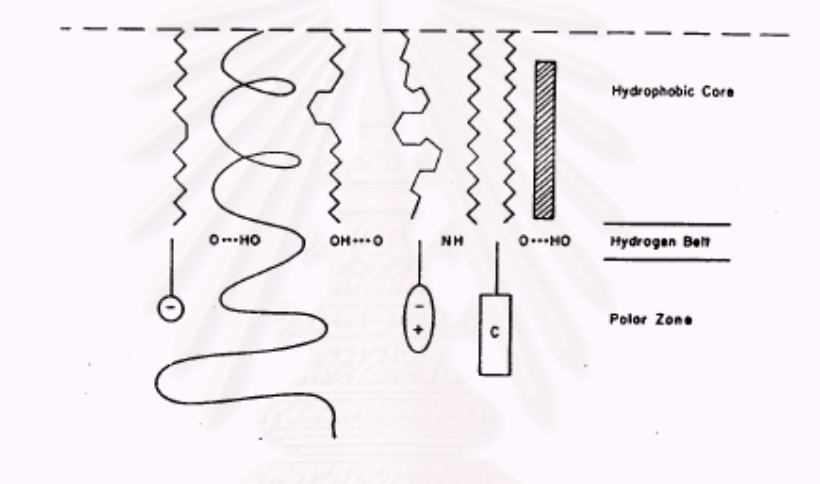

Figure 3 Stratification of a biological membrane.
Zigzag lines represent aliphatic chains, the shade rectangle is cholesterol, the coil is protein, + and – are charged phospholipid heads, C is carbohydrate, O atoms are supplied by the carbonyl groups of phospholipids, NH is from proteins and OH is from cholesterol, water and protein.

The phospholipids are aligned with the glycerol backbone nearly perpendicular to the plane of the membrane and the polar head groups roughly parallel with the membrane surface. At the same time, the hydrocarbon chains tilt relative to the plane of membrane so the chains become closer and maximize Van der Waal interactions as demonstrated in Figure 4 [New, 1990].

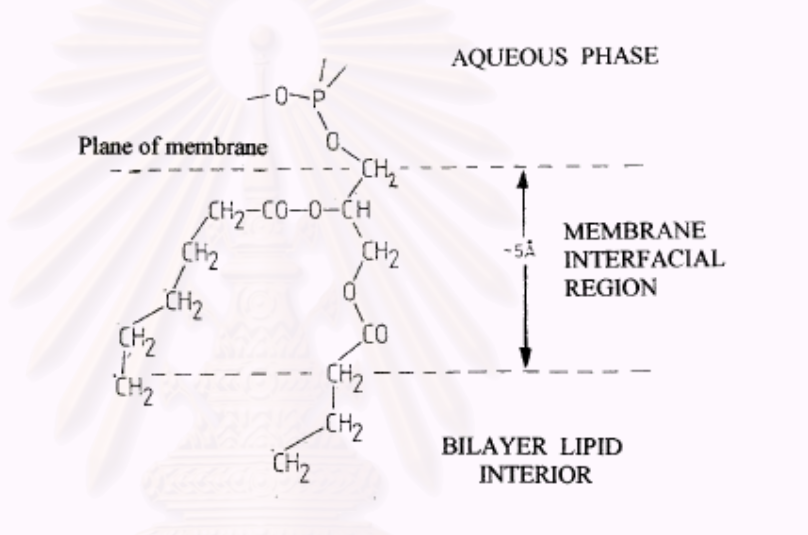

Figure 4 Schematic conformation of phosphatidyl choline.
The diagram shows the orientation of the glycerol bridge approximately perpendicular to the plane of the membrane.

The bilayers maintain their structures by the balance between phospholipid-phospholipid force and phospholipid-water force. This bilayer formation is the basic structure of all biological membranes. Anything that weakens these two forces may disorder and fluidize the membrane structure. The increasing temperature brings about the alteration of hydrocarbon chain conformation and this tends to expand the hydrocarbon chains and then

decreases the bilayer thickness [New, 1990]. The loss of the supported interfacial water may destabilize the membrane and increase the fluidity of the membrane core [Chiou, 1992]. Phospholipid bilayers are extremely stable structures which hydrophobic interactions of hydrocarbon chains lead to the smallest area for water to be in contact with the chains and water is separated from the internal of the bilayer. The phospholipid bilayers will close themselves and form spherical vesicles separating the external surrounding from the internal part, and are called liposomes (Figure 2c) [Devlin, 1997]. Thus, it is reasonable to use liposomes which have membrane structures similar to biological membranes as model membranes. Liposomes can be characterized by their size and shape [New, 1990].

Small unilamellar vesicles (SUV) are the smallest vesicles with about 25 nm diameter. Such this smallest size, the headgroups possibly tolerate as the curvature of the inner layer increases with decreasing radius. Multilamellar vesicles (MLV) are the vesicles having a wide range of size (100-1000 nm) and equal to the dimensions of living cells. They are generally composed of five or more concentric membrane lamellar. Because of the different sizes of liposomes, they require different methods of preparation. In practice, the methods give rather heterogeneous vesicles with a wide distribution of size. Hence, the methods of liposome preparations are characterized according to size in order to interpret the properties correctly.

Reversed micelle (Figure 5) is one type of various aggregation forms of phospholipid presented in bilayer membrane which plays an important role in biological process, e.g. membrane fusion [Walde, 1990]. It is sandwiched between the monolayers of the bilayer where the polar head groups of

phospholipids face into the interior of the micelle and surround a water droplet while the two acyl chains direct outward into the hydrophobic portion of the monolayer.

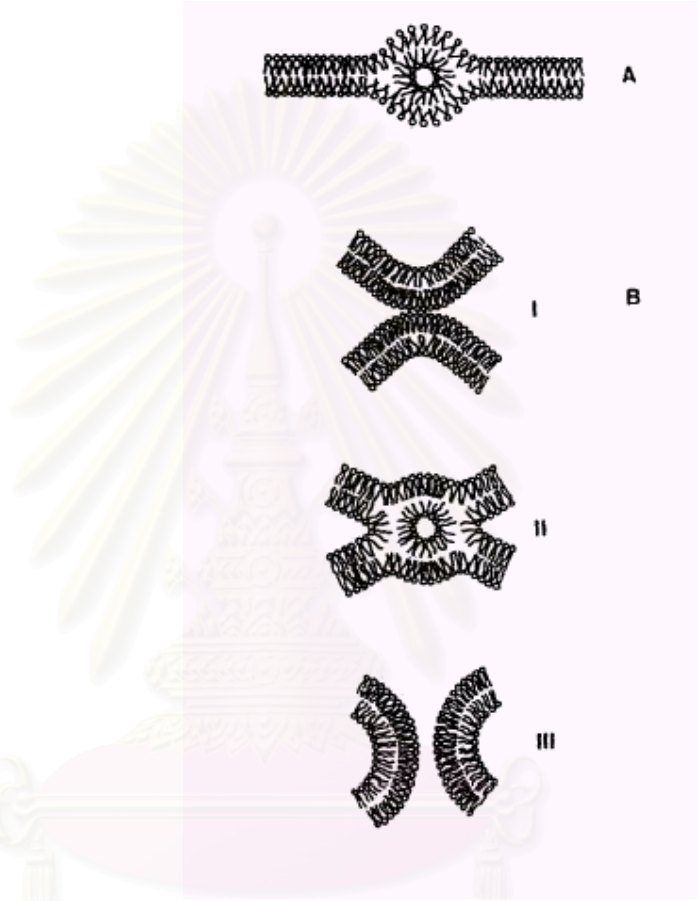

Figure 5 (A) Schematic representation of a reversed micelle sandwiched between the two monolayers of the phospholipid bilayer.
(B) Tentative drawing of a membrane fusion process. I: adhesion, II: joining and III: fission.

Reversed micelles can be formed in many organic solvents, such as chloroform, carbon tetrachloride, benzene and so on. It has been observed that several properties of the reversed micelles depend on the amount of water in the core. The increasing amounts of water to reversed micelles will alter the properties of the inner water itself and also the packing of the phospholipid molecules. The concentration that micelles are first formed in the solution is termed the critical micelle concentration (CMC) which determined from the inflection point of plots of various physical properties of the solution as a function of concentration. The physical properties such as surface tension, conductivity, light scattering intensity and osmotic pressure have been used to determine the CMC. However, there are some particular problems with the reversed micelles. The cooperativity of reversed micelle formation is much smaller than normal micelles (micelles which are formed in aqueous media) and a well-defined CMC can seldom be identified. Many techniques to determine CMC are only applicable in aqueous media. In addition, the CMC loses its significance, since it changes with the water content. It may be stated that the knowledge of phospholipid reversed micelles is to date rather limited and is still wide open for investigation [Walde, 1990].

Infrared Spectroscopic Studies of Biomembranes

Infrared spectroscopy is a method which now plays a significant role of analysis. The field of infrared instrumentation has changed dramatically in the past years. The invention of the Fourier transform algorithm has permitted the development of a new generation of infrared instruments. This results in higher signal-to-noise ratios compared to conventional instruments. One particular advantage of infrared spectroscopy is its nondestructive technique that monitors molecular vibrations and operates on a very short time. In addition, it can give

both qualitative and quantitative results. Because of the enhancement of its sensitivity and specificity, infrared spectroscopy now also works well with quantitative analysis and a very small amount of sample is required for preparation.

The proper sample preparation is essential to the infrared spectroscopy of biological samples. In addition, the infrared absorption of biological samples is hindered by the intense absorption of water as solvent. The way to overcome the problems is to use special infrared techniques, such as attenuated total reflection. Attenuated Total Reflectance Infrared (ATR-IR) spectroscopy is a practical and highly informative technique for the study of biological membranes. This technique can give the information of chemical groups at the water-membrane interface and allow to control and manipulate the conditions such as temperature, pressure and chemical composition of the aqueous phase [Silvestro, 1998]. With this technique, the aqueous suspension of phospholipid vesicles are deposited as films or layers formed by evaporation directly on an appropriate supporting material. The supporting material is a thin, optically transparent crystal of high refractive index (R_f). The IR beam is focused and directed by suitable mirrors into one end of the crystal and strikes the sample-crystal interface. The total internal reflection occurs when the refractive index of the sample is less than that of the crystal. The IR beam penetrated into a few distance (μm) of the sample film before returning to the crystal as illustrated in Figure 6. If the sample absorbs energy at that wavelength, the IR beam will be attenuated. Depending on the incident angle and the length of the crystal, 6-35 reflections can be achieved before the beam reemerges from the crystal to the detector.

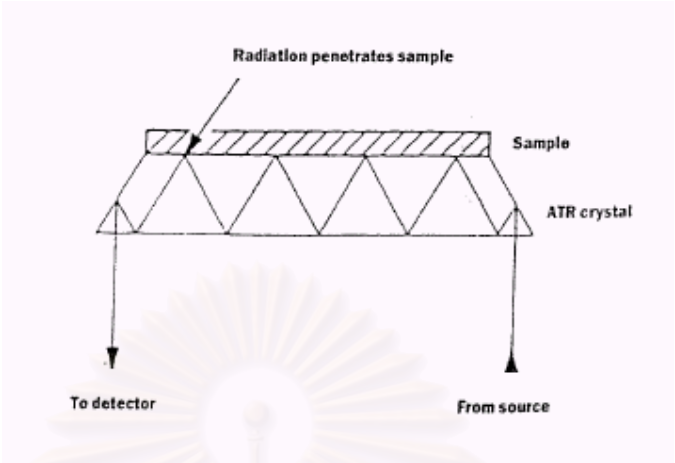

Figure 6 Reflections in an ATR crystal.

The ATR crystal should be IR transparent and has a high refractive index (R_f = 2.5-4.0). The generally used material are germanium (water insoluble, R_f = 4.01, transmission range 3300-400cm^{-1}, very brittle), KRS-5 (soluble in bases, acid insoluble, not hygroscopic, R_f = 2.38, transmission range >260 cm^{-1}), zinc selenide (insoluble in water, organic solvents, dilute acid/base, R_f = 2.4, transmission range >500 cm^{-1}). Most ATR units permit the crystal to be mounted in several positions with different incident angle and number of reflections. This allows selecting the position which optimizes the resulting spectrum [Chapman, 1984]. Moreover, the invention of interferometric Fourier transform infrared spectrophotometer and the availability of data-handling techniques have opened up new ways for the study of aqueous biological membranes. Since the output of modern spectrophotometers is digitized, the data processing is simplified and can be performed on computer. The technique of band narrowing or deconvolution is very useful for the study of bands comprised of more than one component. The deconvoluted spectra have narrower bands but correct integrated intensities and frequencies. Difference technique can be

used to subtract the solvent band from the spectrum. These evidences indicate that infrared spectroscopy is now being used effectively for biological studies as a routine technique and aqueous sample can be analyzed successfully [Casal, 1984].

According to Casal et al.,1984, the infrared spectra of phospholipid in biological membrane can be separated into two regions which are the molecular vibrations of the hydrophobic hydrocarbon tail and those of the hydrophilic head group as follow,

Acyl chains

CH_2 antisymmetric stretching	2920 cm^{-1}
CH_2 symmetric stretching	2850 cm^{-1}
Terminal CH_3 asymmetric stretching	2956 cm^{-1}
Terminal CH_3 symmetric stretching	2870 cm^{-1}

Head group

C=O stretching	1750-1700 cm^{-1}
sn-1 C=O stretching (closer to the lipid core)	1742 cm^{-1}
sn-2 C=O stretching (closer to the polar head)	1725 cm^{-1}
C-O stretching	1170 cm^{-1}
$N^{+}(CH_3)_3$ asymmetric stretching	970 cm^{-1}
P=O antisymmetric stretching	1250 cm^{-1}
P=O symmetric stretching	1085 cm^{-1}

Wong et al. (1988) compared the infrared sprctra of anhydrous and fully hydrated dipalmitoyl phosphatidylcholine (DPPC) in water (Figure 7). The

frequency of P=O asymmetric stretching band shifted downward from 1262 cm^{-1} in the anhydrous sample to 1222 cm^{-1} (marked by dots) in the fully hydrated sample. The decrease of 40 cm^{-1} in the P=O frequency upon hydration of DPPC was indicative of hydrogen bonding between water and the P=O groups in fully hydrated DPPC. Upon hydration, the C=O stretching band broadened as shown in Figure 8. In the deconvoluted spectra of nearly anhydrous DPPC sample, the C=O stretching bands could be resolved into three components at 1744.3, 1737.5 and 1728.2 cm^{-1} (marked by dots). The band at 1744.3 cm^{-1} was the C=O stretching mode of the free sn-1 C=O group while the band at 1737.5 and 1728 cm^{-1} were the C=O stretching mode of the free and hydrogen-bonded sn-2 C=O groups, respectively. In fully hydrated DPPC, the C=O stretching band could only be resolved into two broad bands at the same frequencies as those of the free sn-1 C=O group and the hydrogen-bonded sn-2 C=O group.

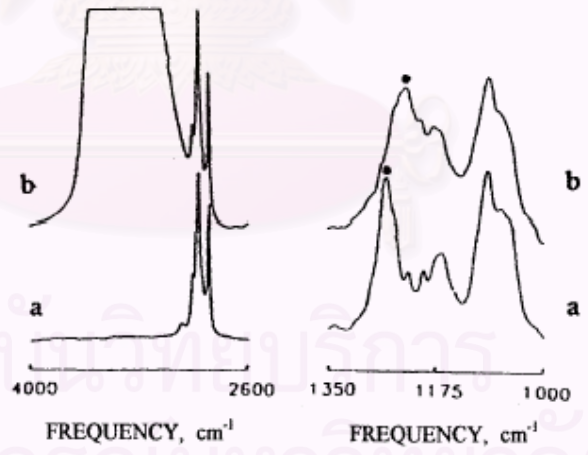

Figure 7 Infrared spectra of (a) anhydrous DPPC and (b) fully hydrated DPPC in the spectral regions 4000-2600 cm^{-1} (OH and CH stretching band) and 1350-1000 cm^{-1} (P=O stretching band).

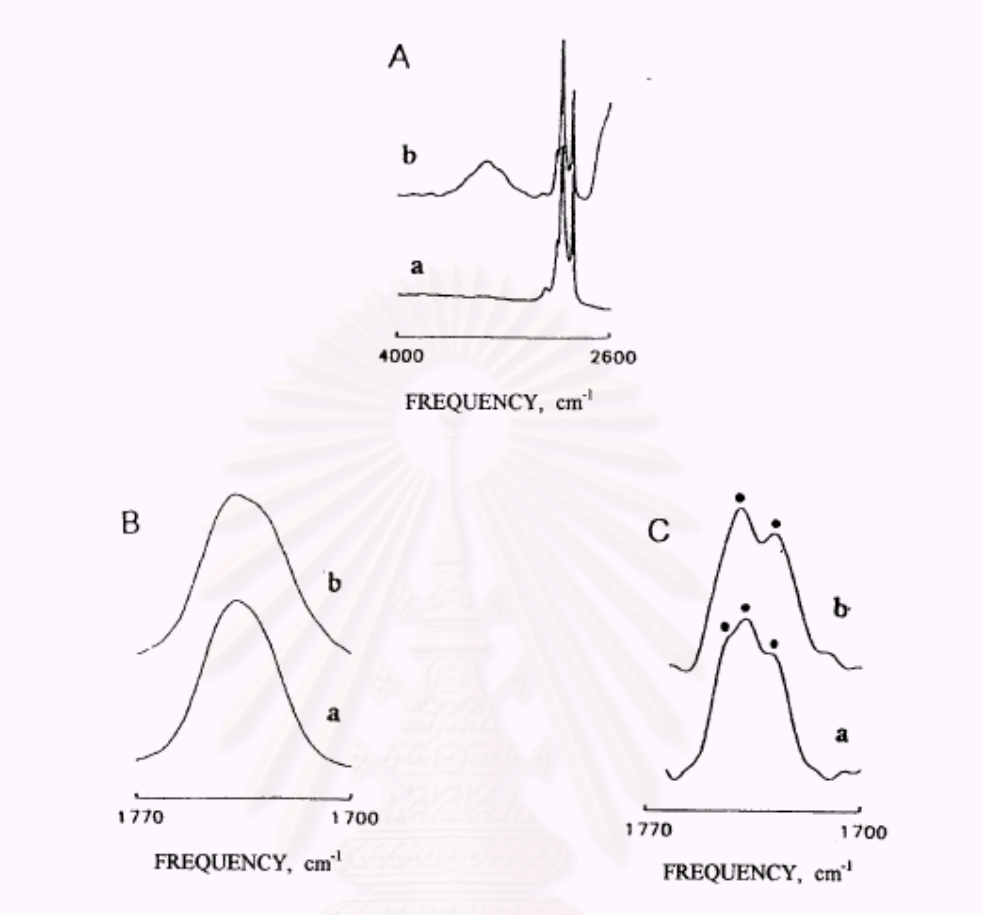

Figure 8 Infrared spectra of (a) nearly anhydrous DPPC and (b) fully hydrated DPPC.
(A) OH and CH stretching band, (B) C=O stretching band and (C) deconvoluted C=O stretching band.

Mushayakarara et al. (1986) reported that the pressure at 20 kbar could free the lipid carbonyl groups from the hydrogen bonding to water. In this experiment, triacetin, triacetyl glycerol, was used because of its ester carbonyl moiety and solubility in water. The carbonyl band of triacetin in deuterium oxide (D_2O) moved downward from 1728 cm^{-1} at atmospheric pressure to 1725 cm^{-1} at

15 kbar as shown in Figure 9. This diminution of frequency indicated that the increasing pressure strengthened the hydrogen bond. However, at the pressure of 20 kbar, the frequency of C=O stretching band moved upward to 1744 cm^{-1} which was the free C=O stretching band. Then, the frequency increased upon applying pressure between 20-30 kbar. The shift of wavenumber to higher frequency at 20 kbar showed that water was withdrawn from the interfacial zone by the suction of stronger water-water association. Water preferentially bound to itself and withdrew the bound water molecules from the interfacial region of triacetin. In fact, this pressure corresponded to the pressure which ice VII was formed. The water molecules experienced a large increase in hydrogen bonding to the adjacent water molecules upon ice VII formation. This observation supported the conclusion that pressure could induce dehydration of the lipid interface.

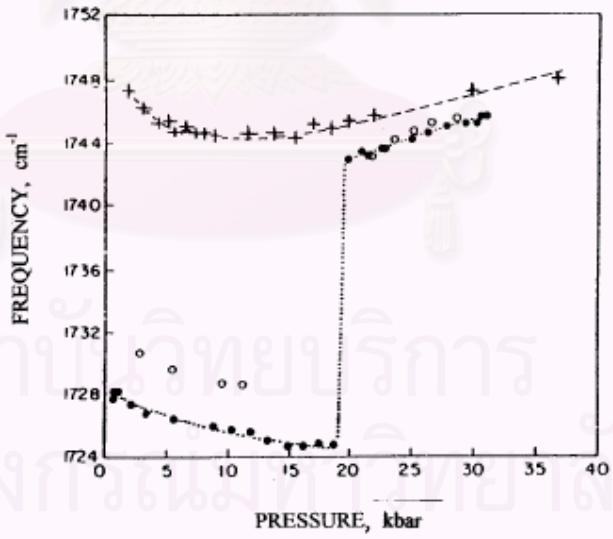

Figure 9 Pressure dependence of the C=O stretching frequencies of triacetin (+) and of its solution in D_2O (●) and H_2O (o).

Chiou et al. (1992) studied the effects of alcohols on the hydrogen bonding of DPPC by Fourier transform infrared (FT-IR) spectroscopy in water-in-CCl_4 reversed micelles. In the presence of ethanol, a new band appeared at 3680 cm^{-1} which was assigned as free O-H stretching band of water molecule (Figure 10).

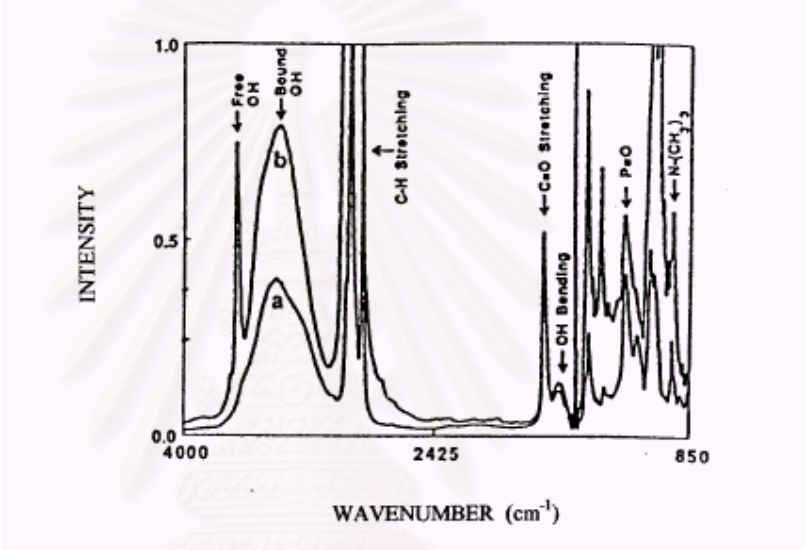

Figure 10 Difference infrared spectra of DPPC-H_2O-CCl_4 reversed micelle after subtraction of the CCl_4 absorption bands. (a) Control without ethanol and (b) with 0.10 M of ethanol.

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From the difference spectrum in Figure 11, the negative value of peak at 3635 cm^{-1} which was the ethanol free OH stretching band indicated loss of the free ethanol by binding to DPPC as well as the broad band at 3235 cm^{-1} was the bonded OH stretching band of ethanol forming hydrogen bond with DPPC. P=O stretching band was shifted by ethanol to the lower frequency with increasing the intensity and broadening the band. This result indicated that ethanol bound to phosphate group in competition with water therefore alcohols weakened the water-DPPC interactions and dehydrated the phosphate surface by competitive binding.

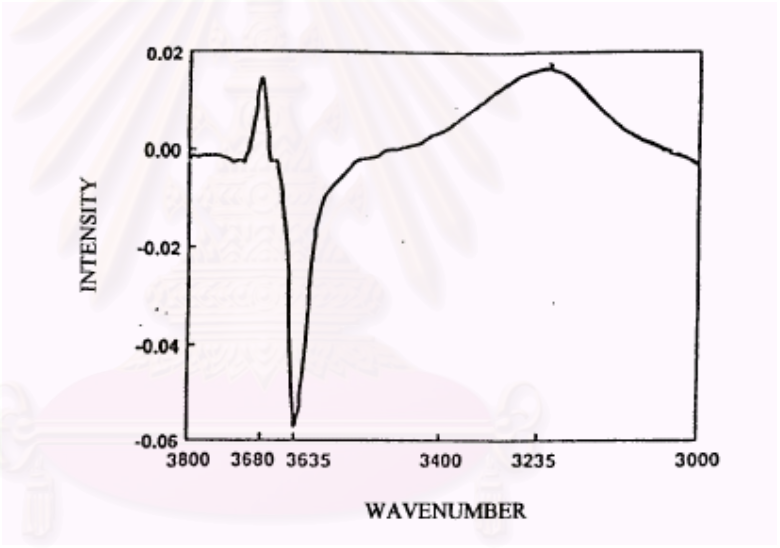

Figure 11 Difference infrared spectrum between 0.15 M ethanol interacted with the reversed micelle and ethanol in CCl_4.

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The DPPC molecule has two ester groups which are overlapped each other and can be observed only one band at 1734 cm^{-1}. When alcohols were added, the intensity of this band decreased and the band was shifted slightly to the higher frequency. The C=O stretching band of partially hydrated DPPC can be deconvoluted into three components: the free sn-1 C=O band, the free sn-2 C=O band and the hydrogen-bonded sn-2 C=O band. The free sn-1 C=O group is located closer to the lipid core whereas the free sn-2 C=O group is located closer to the polar head. The hydrogen-bonded sn-2 C=O group is the hydrated sn-2 C=O group. The deconvoluted C=O spectrum of these three groups are at 1746, 1738 and 1729 cm^{-1} (marked by dots), respectively (Figure 12). Ethanol increased the intensity ratio between the free and the hydrogen-bonded sn-2 C=O bands and the ratio increased more when the lipid-to-water ratio was higher. This increased ratio and the decreased intensity of the C=O band demonstrated the dehydration effect of alcohols; however, alcohols themselves could not directly form hydrogen bonds with C=O moieties because of steric hindrance in penetrating into this level. In addition, the constancy of the free sn-1 C=O band indicated that alcohols could not interact with this group which was closer to the hydrophobic region of the membrane. The mentioned effects of alcohols could conclude that DPPC-water complex was partially replaced by DPPC-alcohol complex. The bound water molecules were released from the phosphate and the sn-2 C=O moieties when alcohols were added. The bound alcohols created a new broad band.

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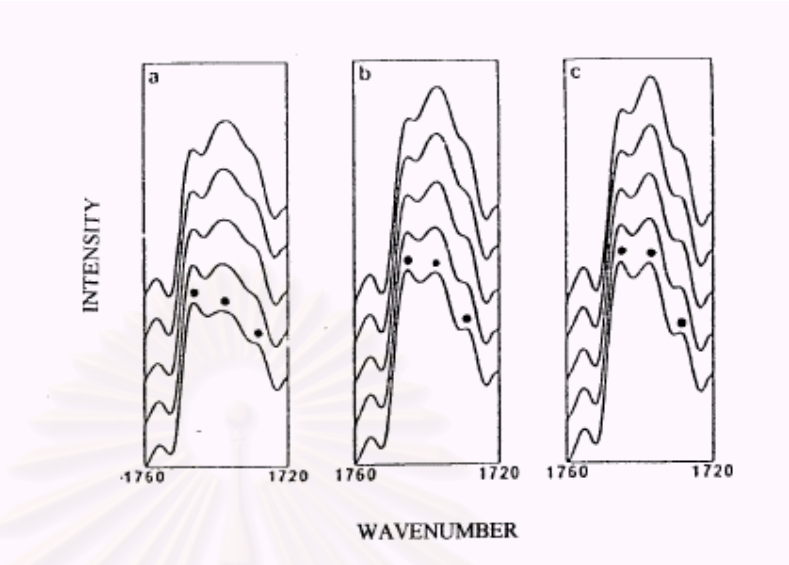

Figure 12 Deconvoluted infrared spectra of the C=O stretching band at various DPPC/water ratios and ethanol concentrations. DPPC/water ratio: (a) 2:36, (b) 4:36 and (c) 6:36. Ethanol concentrations from the bottom upward are: 0, 0.025, 0.050, 0.075 and 0.10 M.

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Tsai et al. (1990) compared polar halothane and enflurane with apolar carbon tetrachloride concerning their effect on water-membrane interaction by ATR-IR spectroscopy and DPPC multilamellar vesicle (MLV) was used as a model membrane. It was previously reported that the main hydration region of DPPC membrane was the phosphate moiety. In addition, the choline head group signal was unaffected by anesthetic disturbance and this group was little hydrated by water. Thus, the ratio of the P=O stretching band area to the $N^{+}(CH_3)_3$ stretching band area was used to evaluate the hydration of the membrane and represented the change in phosphate moiety. At the lipid/anesthetic mole ratio 2:1, this absorption band-area ratio increased from 3.8 of control to 4.8, 4.6 and 4.2 of halothane, enflurane and carbon tetrachloride, respectively. Halothane and enflurane had greater effect on the absorption band-area ratio than carbon tetrachloride. The deconvoluted carbonyl spectrum of the fully hydrated diacyl phospholipid membrane had two components at 1742 cm^{-1} and 1727 cm^{-1}. The 1742 cm^{-1} peak was the free sn-1 C=O group in the hydrophobic region and the 1727 cm^{-1} peak was the hydrogen-bonded sn-2 C=O group close to the polar head region. Figure 13 showed the deconvoluted C=O spectra with anesthetics. Three peaks were resolved in case of halothane (at 1744, 1735 and 1728 cm^{-1}) and enflurane (at 1740, 1734 and 1729 cm^{-1}). On the other hand, there were only two peaks at 1739 and 1728 cm^{-1} for carbon tetrachloride addition. This result showed that halothane and enflurane brought about the formation of the free sn-2 C=O peak by disruption hydrogen bonding to water (at 1735 and 1734 cm^{-1}, respectively) but no free sn-2 C=O peak was found in case of carbon tetrachloride addition.

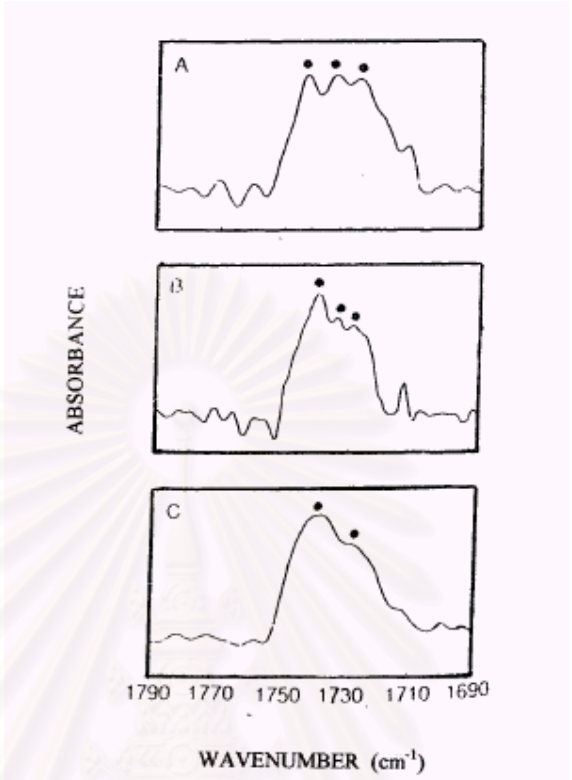

Figure 13 Deconvoluted infrared spectra of the C=O stretching band of DPPC MLV/anesthetic samples.
(A) halothane, (B) enflurane and (C) carbon tetrachloride.

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Ueda et al. (1994) suggested that the primary action site of local anesthetics was the lipid-water interface. This study was analyzed by Fourier transform infrared spectroscopy in water-in-oil reversed micelle. Local anesthetics used in this study were tetracaine, dibucaine and lidocaine. Figure 14 showed the effect of lidocaine on DPPC-H_2O-CCl_4 reversed micelles between 3700-3050 cm^{-1}. To identify the bands, the spectrum was deconvoluted and showed the bound NH stretching of local anesthetic-lipid band at 3384 cm^{-1} and the free OH stretching of water-anesthetic band at 3480 cm^{-1}.

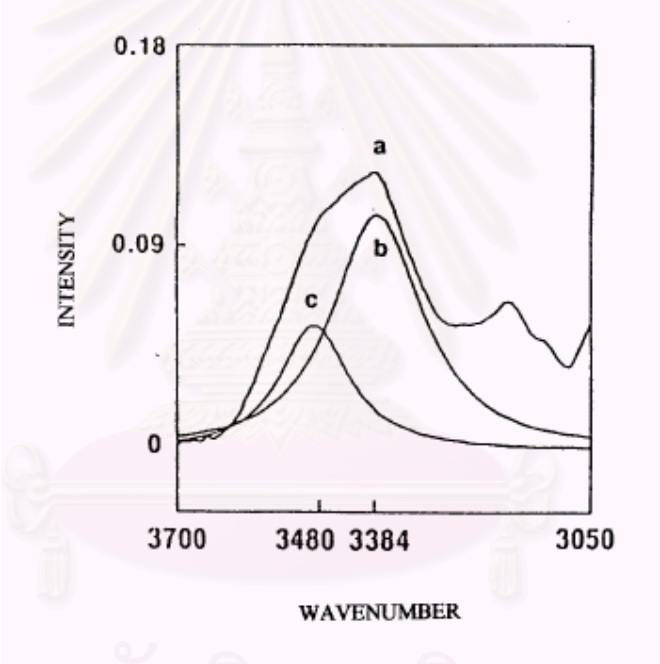

Figure 14 Deconvoluted infrared spectra of the lidocaine-DPPC-H_2O-CCl_4 reversed micelle. The lidocaine-CCl_4 and DPPC-H_2O-CCl_4 spectra were subtracted. (a) original infrared spectrum, (b) bound NH stretching of lidocaine-DPPC deconvoluted band and (c) free OH stretching band of the water-lidocaine deconvoluted band.

Upon increasing of lidocaine concentration, the P=O stretching band shifted downward from 1236 cm^{-1} to 1233 cm^{-1}. Since P=O group could form hydrogen bond with molecules containing OH or NH group, as a result P=O stretching band shifted to lower frequency. Thus, this demonstrated the dehydration effect and the occurrence of replacement by local anesthetic molecules in the hydrophilic head group region. The C=O stretching band appeared as a single peak at 1734 cm^{-1} and lidocaine shifted this band to 1740 cm^{-1}. Figure 15 illustrated deconvoluted spectra of C=O stretching bands. When lidocaine concentration increased, the intensity of free sn-2 C=O stetching band (1736 cm^{-1}) increased in contrast with hydrated sn-2 C=O stretching band (1728 cm^{-1}). However, the free sn-1 C=O stretching band (1744cm^{-1}) did not change. The alteration of the C=O stretching band indicated dehydration of this region by releasing hydrogen-bonded water from sn-2 C=O moieties.

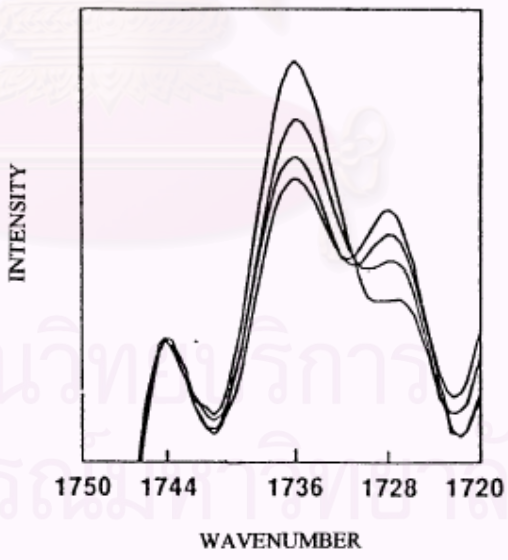

Figure 15 Lidocaine effects on the deconvoluted C=O stretching bands of the DPPC-H_2O-CCl_4 reversed micelle.
The lidocaine concentrations are from the bottom at 1736 cm^{-1} : 0, 3, 5 and 10 mM.

Benzodiazepines

The benzodiazepines are the most important sedative-hypnotics. The effects of the benzodiazepines virtually all result from actions of these drugs on central nervous system (CNS). The major molecular targets of the benzodiazepines are inhibitory neurotransmitter receptors directly activated by the amino acid, GABA. According to the $GABA_A$ receptor hypothesis for benzodiazepine action, benzodiazepines directly bind to the receptor / ion channel complex and allosterically modulate its activity. Binding of benzodiazepines facilitates the process of chloride channel opening with resultant membrane hyperpolarization [Hardman, 1996].

The structure of the benzodiazepines as illustrated in Figure 16 is composed of a benzene ring (A) fused to a seven-membered diazepine ring (B) and all the important benzodiazepines contain a 5-aryl subunit (ring C) and a 1,4-diazepine ring.

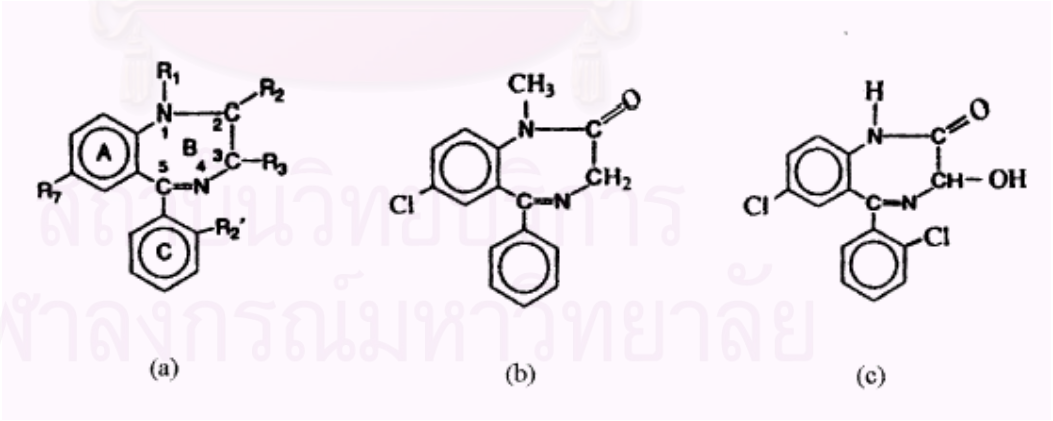

Figure 16 The structure of (a) benzodiazepine, (b) diazepam and (c) lorazepam.

Some studies have pointed out high affinity stereospecific binding site for benzodiazepines in CNS. Several attempts have been carried out to isolate and identify endogenous ligands interacting with the benzodiazepine receptor under physiological conditions. The uncertainties on the nature of the endogenous ligand make very difficult to hypothesize a reasonable scheme of the forces binding benzodiazepines to their receptor. Although these forces can be generally identified in a combination of hydrogen bonding, electrostatic and hydrophobic (e.g. Van der Waals) interactions, only some evidences for a contribution of hydrogen bonding interactions have been obtained. As regards to the hydrogen bond donor-acceptor properties of the benzodiazepine, the most frequently observed acceptor is the oxygen of the carbonyl group and the most common donor is N_1H. The OH group at carbon atom 3 is found to act both as donor and acceptor. Borea et al., 1982 showed the possible role of hydrogen bonding in benzodiazepine-receptor interactions. They concluded that benzodiazepine-receptor interaction determined by a hydrogen bond mechanism was mainly associated with groups which were hydrogen bond donors, the N_1H or OH group at carbon atom 3. Parts of the benzodiazepine molecule suitable for hydrogen bonding interaction were $-N_1H-C_2O-$ and $-C_3(OH)-N_4=$ groups. As the relative importance of these two groups, it appeared to be in favor of the $-N_1H-C_2O-$ group because there was the greatest electron withdrawing effect caused by electronegative substituents in position 7 on the hydrogen linked to the N_1 atom.

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Kurishingal et al., 1992 proposed the benzodiazepine-induced changes in biomembrane fluidity by fluorescence polarization measurement using DPH as a fluorescent probe. The results showed that chlordiazepoxide and diazepam were capable of fluidizing the membrane at low, physiologically-relevant concentrations. As the concentration was increased, there was an apparent ordering of the membrane once more (Figure 17).

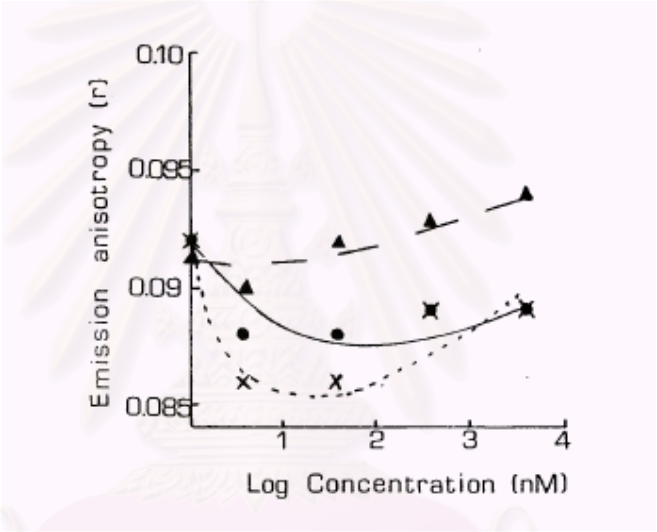

Figure 17 The effect of chlordiazepoxide (▲), diazepam (●) and Ro 15-1788 (×) on the steady state fluorescence anisotropy of DPH-labelled egg yolk phosphatidylcholine membranes.

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Since, one site of action of benzodiazepines is on biological membrane, thus, there is an enthusiasm to investigate existence of hydrogen bond interaction between benzodiazepines and membrane and also the correlation between amount of drug and degree of hydrogen bonding. The achieved results may be related to effectiveness of benzodiazepine in medical therapy.

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CHAPTER III

MATERIALS AND METHODS

Instruments

1. Ultrasonic bath (Elma)
2. Incubator (Hotpack)
3. Fourier Transform Infrared spectrophotometer (Perkin Elmer Spectrum 2000)

Materials

1. Phospholipids

   1.1 Egg phosphatidylcholine (EPC)

   EPC isolated from egg yolks was the gift from Dr. Usa Glagasigij (Department of Pharmaceutical chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University). Its purity is 92.45% determined by Barlett assay.

   1.2 Dipalmitoyl phosphatidylcholine (DPPC)

   DPPC was purchased from Sigma Chemical Co.

2. Benzodiazepine drugs

   Benzodiazepine drugs used in this experiment were diazepam and lorazepam. They were the gift from Pharmasant Laboratories Co., Ltd.

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3. Deuterium oxide (D_2O) (Merck)
4. Dried chloroform, prepared by passing chloroform through activated aluminium oxide column to eliminate water.
5. Other reagents used in the experiment were of analytical grade.

Methods

1. Effect of benzodiazepine drugs on reversed micelles

   Preparation of reversed micelles

   Reversed micelles were prepared by dispersing EPC or DPPC in chloroform to obtain 15 mM concentration of phospholipid. Then, deuterium oxide was added to the solution of phospholipid with a micro syringe to obtain 90 mM concentration of deuterium oxide. The phospholipid/D_2O/$CHCl_3$ mixture was sealed and then sonicated in ultrasonic bath until the dispersion became clear (about 10 minutes). The procedure was repeated by increasing the deuterium oxide concentration to 135 mM.

   Diazepam was prepared in chloroform to obtain 25 mg/ml. The diazepam solution was injected to the reversed micelle with a micro syringe in various concentrations e.g., 4, 8, 12 and 16 mM. Then, the sample was sealed and vortex mixed.

   Lorazepam solution in chloroform with 2.5 mg/ml concentration was treated in the similar manner but with concentrations 1, 2, 3 and 4 mM.

Each of reversed micelle preparation was prepared at least three replications.

FT-IR spectroscopic procedure

About 1.2 ml of the reversed micelle or chloroform was injected into zinc selenide (ZnSe) liquid cell with 0.5 mm spacer. Then, the liquid cell was placed in the FT-IR spectrophotometer and infrared spectrum was recorded. Each sample was scanned 16 times through the frequency range of 4,000-650 cm^{-1}. The resolution was 4 cm^{-1} with interval 1 cm^{-1}.

Data analysis

Peak position, peak height (peak intensity) and peak area were measured after subtraction by chloroform blank. After that deconvoluted spectrum was obtained by using Perkin Elmer Software with constant parameter settings (gamma=2 and length=10).

2. Effect of benzodiazepine drugs on liposomes

Preparation of multilamellar vesicles (MLV)

EPC or DPPC was dissolved in chloroform and then dried in a rotary evaporator. The dried phospholipid film was purged with nitrogen to remove the trace of chloroform. The multilamellar vesicle was prepared by adding an appropriate amount of D_2O to the dried phospholipid film to obtain 4 mM concentration of phospholipid. Then, the phospholipid suspension was vigorously vortexed at few degrees higher temperature above the main transition temperature of the phospholipid (room temperature for EPC and 51° C for DPPC) for exactly

25 minutes. At this step, a homogeneous milky white suspension was formed. The multilamellar vesicle was purged with nitrogen and sealed. Finally, the size of multilamellar vesicle was determined by electron microscopy.

Diazepam was dissolved in chloroform to obtain 6 mg/ml and the solution was added in various concentrations e.g., 0.2, 0.5, 0.7 and 1.0 mM to the phospholipid in chloroform. Multilamellar vesicle containing drug was performed as described above.

Lorazepam was also studied but its concentration in chloroform was 3 mg/ml and final concentrations in multilamellar vesicle were 0.5, 1.0, 1.5 and 2.0 mM.

Each of the multilamellar vesicle formulations was prepared at least three replications.

Preparation of small unilamellar vesicles (SUV)

Small unilamellar vesicle was prepared by bath sonication. A proper amount of D_2O was added to dried phospholipid film of EPC or DPPC in test tube to obtain 4 mM concentration of phospholipid. The phospholipid suspension was vigorously vortexed at few degrees higher temperature above the main transition temperature of phospholipid (room temperature for EPC and 51° C for DPPC) until all the phospholipid film was completely removed from the wall of the tube. The obtained milky white suspension was purged with nitrogen and sealed. Then, it was sonicated in an ultrasonic bath using the water temperature few degrees

above the main transition temperature of phospholipid for 30 minutes with occasional shaking. At this step, a clear transparent solution was formed and the size of small unilamellar vesicle was determined by electron microscopy.

Effect of diazepam and lorazepam on small unilamellar vesicles was studied with the same concentrations of drugs as earlier described in multilamellar vesicle.

Each of the small unilamellar vesicle preparations was prepared at least three replications.

FT-IR spectroscopic procedure

The absorption infrared spectra were measured by using attenuated total reflectance method with ZnSe as ATR crystal. About 0.4 ml of the vesicle was layered on the ZnSe crystal trough plate and excessive D_2O of the vesicle was evaporated in an incubator. The thin film was kept in a desiccator for 5 minutes in order to cool down to room temperature before recording infrared spectrum. Each sample was scanned 16 times through the frequency range 4000-650 cm^{-1}. The resolution was 8 cm^{-1} with interval 1 cm^{-1}.

Data analysis

Peak position, peak height (peak intensity), peak area and deconvoluted spectrum (gamma=3 and length=45) were obtained as mentioned above in reversed micelle.

CHAPTER IV

RESULTS AND DISCUSSION

Effect of Benzodiazepine Drugs on Reversed Micelles

The difference spectra shown in Figure 18(a) and 19(a) were obtained after subtraction of the absorption bands of chloroform from those of the DPPC and EPC-D_2O-$CHCl_3$ reversed micelles (15:90 mM), respectively. The frequencies of the major functional groups were:

CH_2 antisymmetric stretching band of acyl chains at	2927 cm^{-1}
CH_2 symmetric stretching band of acyl chains at	2854 cm^{-1}
OH stretching band of traces of bonded water at	3391 cm^{-1}
OD stretching band of bonded D_2O at	2491 cm^{-1}
C=O stretching band of ester group at	1733 cm^{-1}
P=O stretching band of phosphate moiety at	1242 cm^{-1}
and $N^+(CH_3)_3$ stretching band of choline head at	970 cm^{-1}

These assignments agree well with the corresponding bands in phospholipid spectrum reported by Casal et al. (1984). In this study, EPC was selected as the natural phospholipid with its major phospholipid components such as 39% C18:1, 27% C16:0, 14% C18:2, 11% C18:0 and 9% other lipids by weight [Lentz, 1980]. DPPC, a synthetic phospholipid, was selected because of its well known molecular configuration and also the major component in EPC.

Figure 18(b) and 19(b) showed the difference spectra of DPPC and EPC in chloroform, respectively. As compared with the spectra of reversed micelle, the P=O stretching bands of both phospholipid solutions in chloroform were at 1254 cm^{-1} which were higher than that of reversed micelles (Figure 20). This result indicated that P=O groups at hydrophilic region of the reversed micelle were hydrated with D_2O and the hydrogen bond formation shifted them to lower frequencies. The OH stretching band of water at 3390 cm^{-1} in phospholipid spectrum might originate from traces of water contaminating in both phospholipids. Thus, D_2O was used to prepare the reversed micelle, then, hydration effect if occurred, was in different frequency, apart from that of H_2O. In order to study degree of hydration, the reversed micelles were prepared in two different hydrations, 15:90 and 15:135 mM (phospholipid:D_2O). Ueda et al. (1994) reported that at the 6:36 mM of phospholipid:water ratio (15% by weight water:phospholipid), all water molecules were bound to the head group as seen by OD stretching band of bonded D_2O at 2491 cm^{-1}. In present study, the intensity of OD stretching band of bonded D_2O increased upon increasing D_2O concentration as demonstrated in Figure 21 with shift of frequency from 2493 cm^{-1} (DPPC:D_2O = 15:90 mM) to 2497 cm^{-1} (DPPC:D_2O = 15:135 mM) in contrast with free D_2O at 2690 cm^{-1} [Chiou, 1990]. This indicated that all D_2O molecules at high concentration (135 mM) were fully hydrated micelles.

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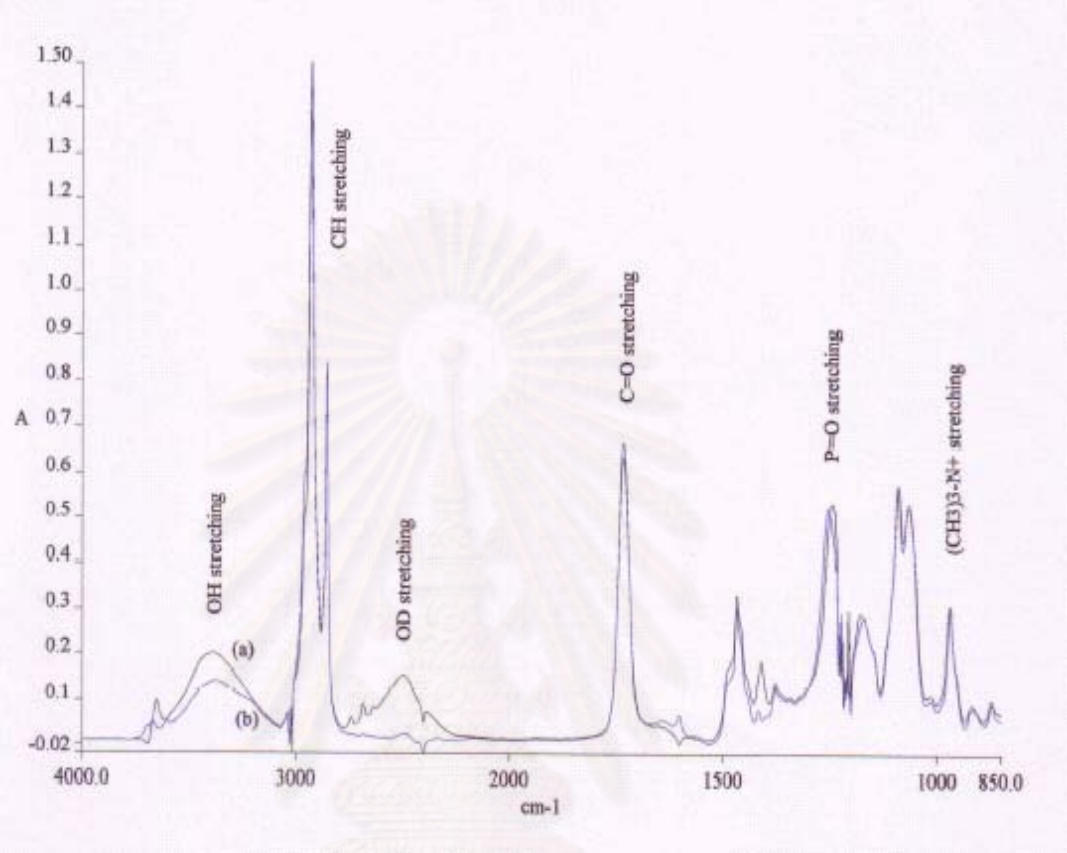

Figure 18 IR difference spectra of (a) DPPC-D_2O-$CHCl_3$ reversed micelle (15:90 mM) and (b) DPPC in $CHCl_3$ (15 mM) after subtraction of the $CHCl_3$ absorption bands.
The IR spectra were prepared by liquid seal cell technique using 0.5 mm spacer.

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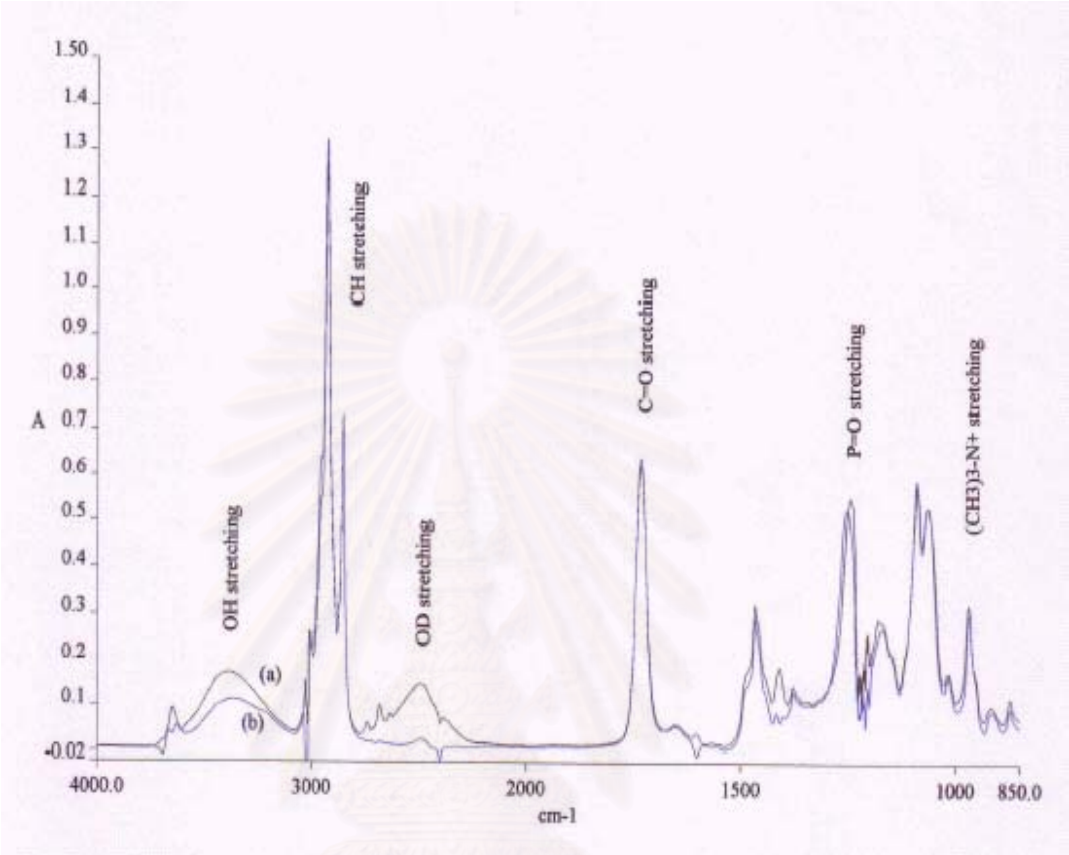

Figure 19 IR difference spectra of (a) EPC-D_2O-$CHCl_3$ reversed micelle (15:90 mM) and (b) EPC in $CHCl_3$ (15 mM) after subtraction of the $CHCl_3$ absorption bands.

The IR spectra were prepared by liquid seal cell technique using 0.5 mm spacer.

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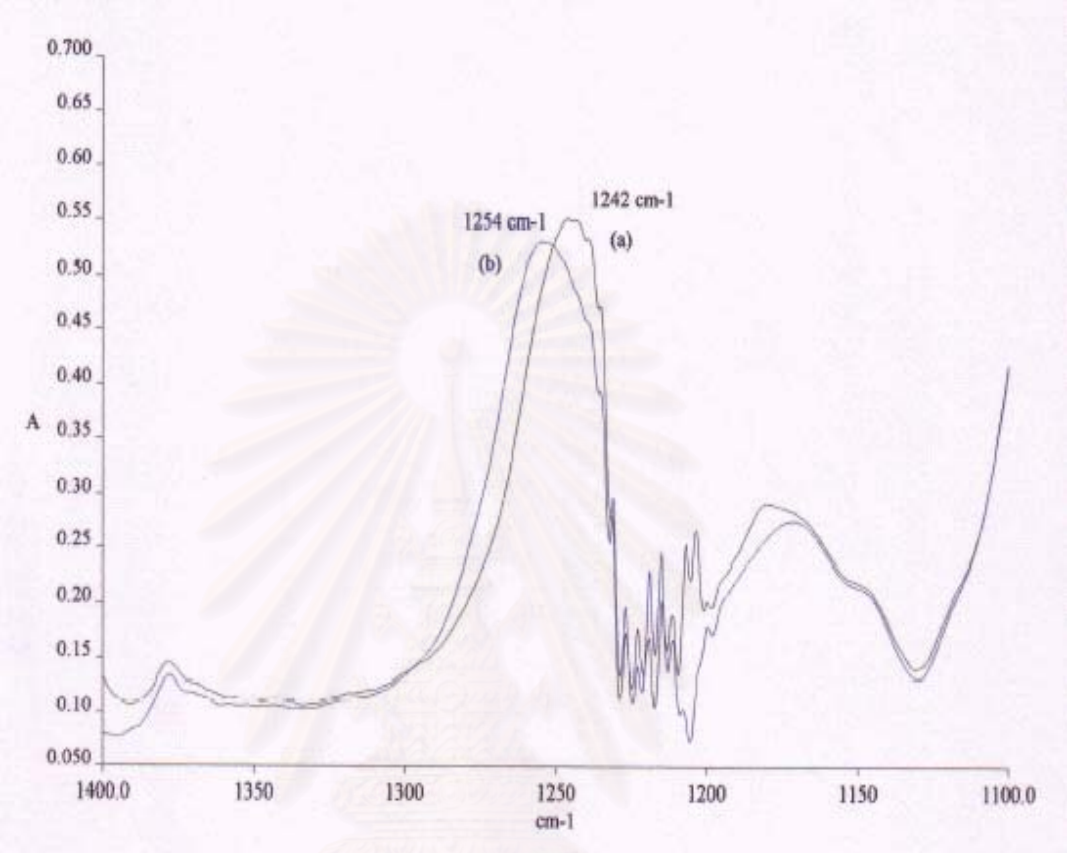

Figure 20 IR difference spectra of P=O stretching band after subtraction of the $CHCl_3$ absorption bands.
(a) EPC-D_2O-$CHCl_3$ reversed micelle (15:90 mM).
(b) EPC in $CHCl_3$ (15 mM).
The IR spectra were prepared by liquid seal cell technique using 0.5 mm spacer.

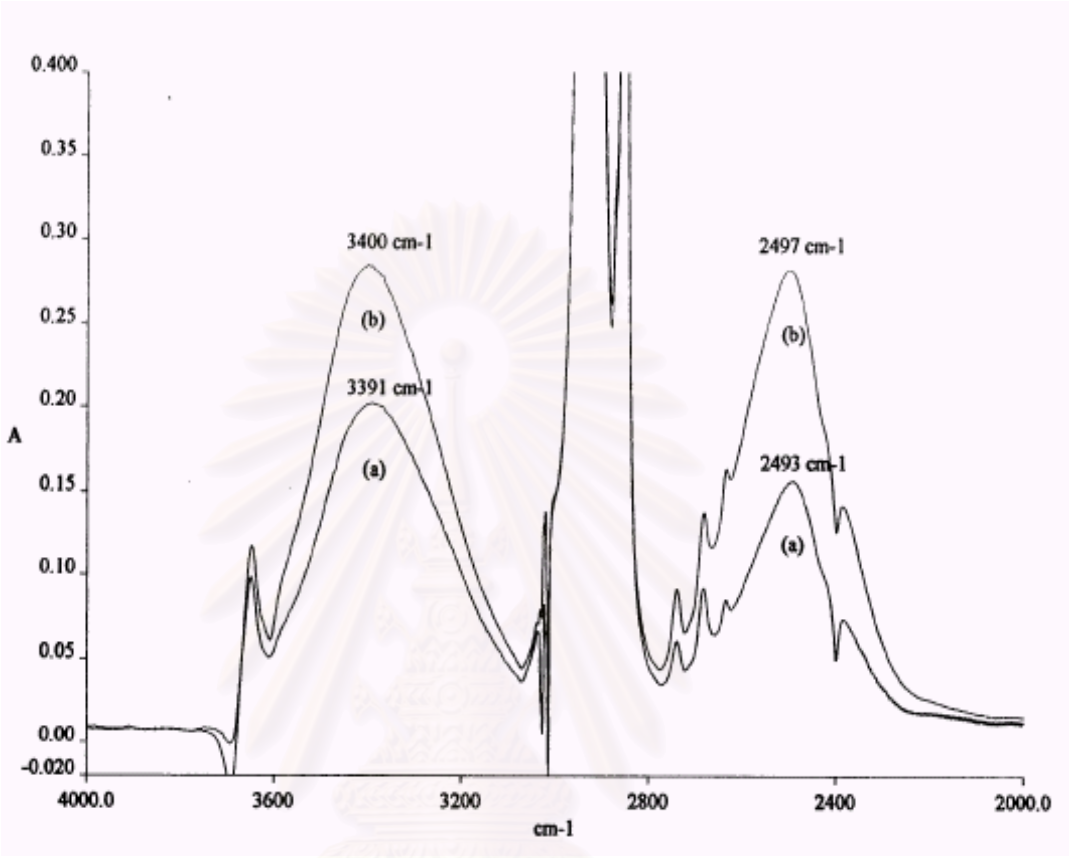

Figure 21 Effect of D_2O hydration on IR difference spectra of DPPC-D_2O-$CHCl_3$ reversed micelle.
Concentration of DPPC to D_2O, by mM. (a) 15:90; (b) 15:135. The IR spectra were prepared by liquid seal cell technique using 0.5 mm spacer.

Benzodiazepine drugs, diazepam and lorazepam were selected as drugs of choice to study their effects on reversed micelle because both drugs had functional groups that could form hydrogen bonds e.g., C=O group of diazepam and C=O, OH and NH group of lorazepam. Drug concentration was varied depending on its solubility in chloroform e.g., 4 to 16 mM for diazepam and 1 to 4 mM for lorazepam. As shown in Figure 22, end of C=O stretching bond of diazepam (1676 cm^{-1}) slightly overlapped with C=O stretching band of ester group of DPPC (1733 cm^{-1}) and its intensity increased upon increasing concentration of drug. On the contrary, enhancement of lorazepam concentration (Figure 23) broadened C=O stretching band of DPPC due to closely frequency value between C=O stretching band of DPPC and that of lorazepam (1705 cm^{-1}).

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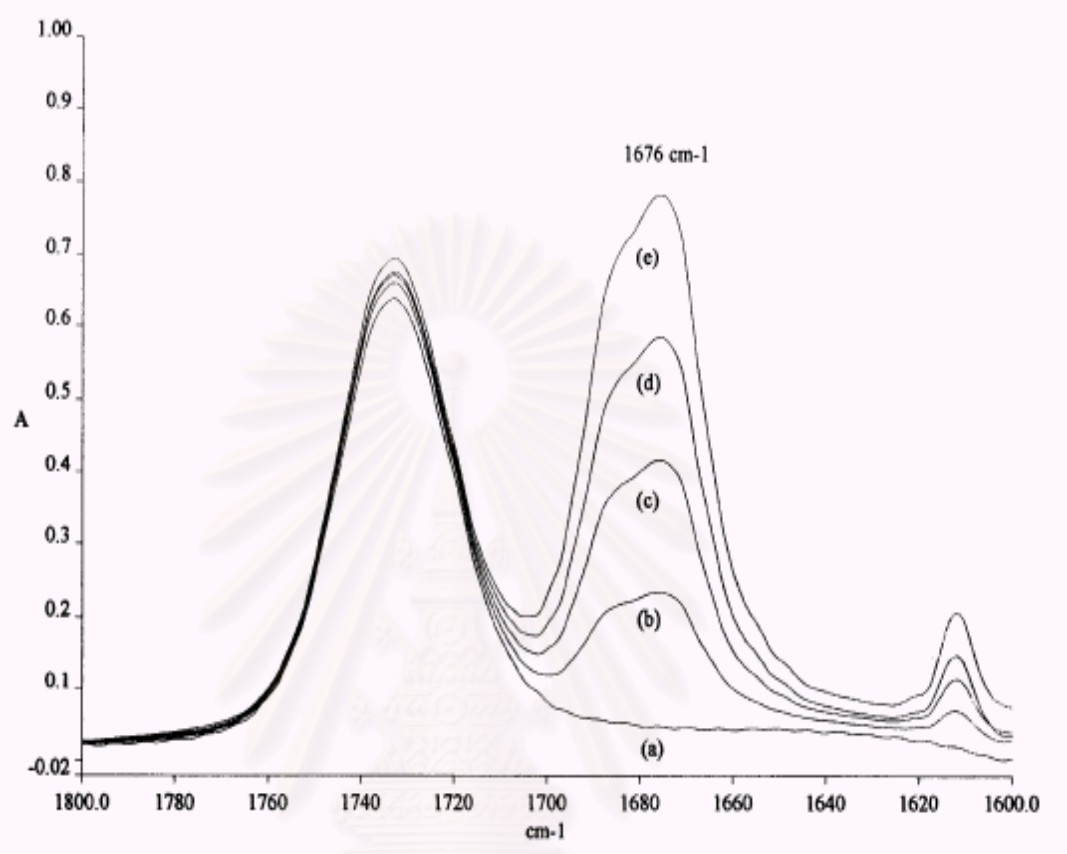

Figure 22 Effect of diazepam concentrations on IR difference spectra of DPPC-D_2O-$CHCl_3$ reversed micelle (15:90 mM).
(a) 0 mM; (b) 4 mM; (c) 8 mM; (d) 12 mM; (e) 16 mM.
The IR spectra were prepared by liquid seal cell technique using 0.5 mm spacer.

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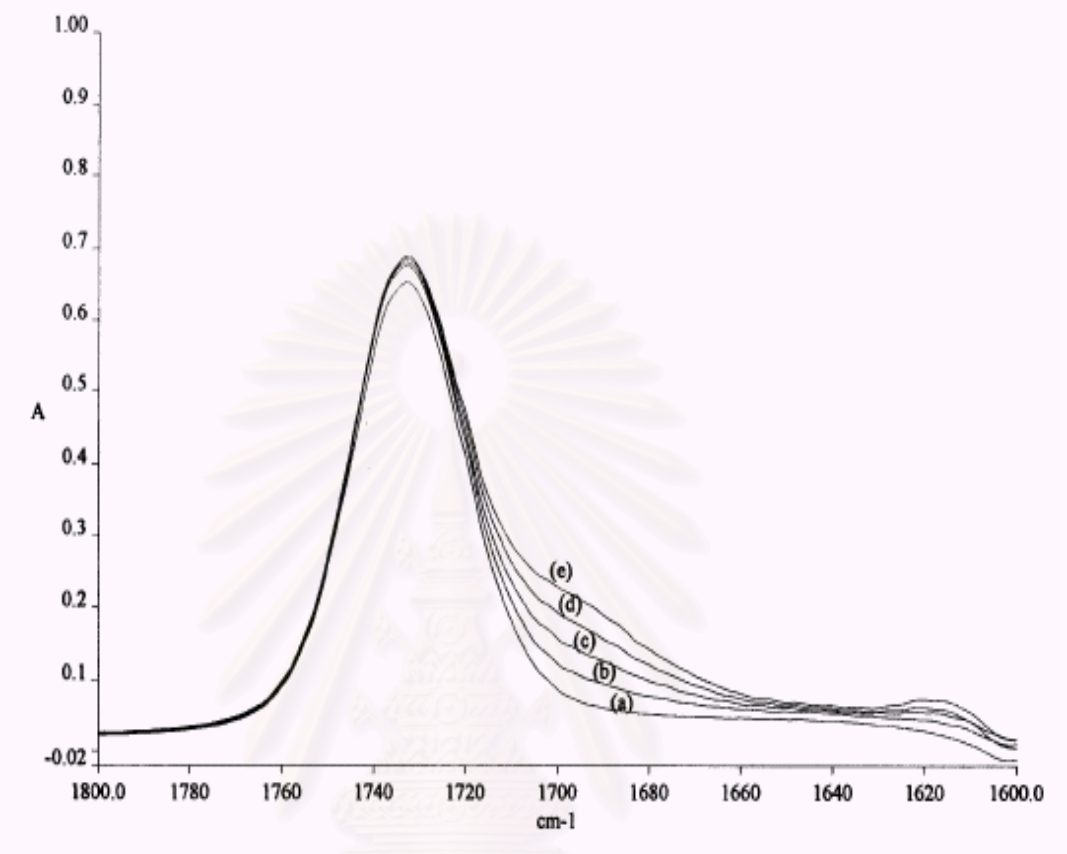

Figure 23 Effect of lorazepam concentrations on IR difference spectra of DPPC-D_2O-$CHCl_3$ reversed micelle (15:90 mM).
(a) 0 mM; (b) 1 mM; (c) 2 mM; (d) 3 mM; (e) 4 mM.
The IR spectra were prepared by liquid seal cell technique using 0.5 mm spacer.

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OH stretching

Most phospholipids can pick up water easily if they expose to atmosphere, then there is a small OH stretching band of water presented in infrared spectrum of phospholipid in chloroform. However, this traces of water form hydrogen bond with phosphate in the head moiety and the polar ester C=O groups in the interfacial region. As a consequence, OH stretching band due to bound water shifts downward from free OH stretching band at about 3680 cm^{-1} to 3391 cm^{-1} as illustrated in Figure 18b and 19b. D_2O itself also contained traces of water as revealed by higher intensity of OH stretching band at 3400 cm^{-1} obtained from DPPC:D_2O reversed micelle at concentration 15:135 mM (Figure 21b) comparing with another micelle 15:90 mM (Figure 21a). Higher shift upward in stretching frequency of bonded water was observed with 15:135 mM reversed micelle (both DPPC and EPC) (Table 1) and this might be due to crowded molecules of water and D_2O around hydrophilic region of phospholipid upon increasing D_2O concentration. As a consequence, higher energy was required to obtain stretching vibrations of bond of molecules.

Upon increasing of diazepam concentration, the frequency of OH stretching band shifted downward from 3391 cm^{-1} to 3374 cm^{-1} when the phospholipid (both DPPC and EPC):D_2O ratio was 15:90 mM (Table 1). The enhancement of intensity and area of OH stretching band were obtained upon increasing diazepam concentration (Table 2). It is well known that hydrogen bond can alter the force constants of both proton donor group and proton acceptor group by an elongation of the chemical bonds of these groups. Thus, the stretching frequencies shift downward usually with increased intensity and band broadening [Wong, 1988 and Siverstein, 1998]. These indicated that diazepam formed hydrogen bond with OH group of traces of bonded water

which might be obtained by releasing of water from its binding site in phospholipid or formed continuous hydrogen bond with bonded water. However, in case of releasing water, this hydrogen bond interaction might be stronger than hydrogen bonding between water and phospholipid since lower frequency was observed.

The enhancement of intensity and area observed in EPC reversed micelle were more obvious than that DPPC reversed micelle and were concentration dependence. The explanation might be due to difference in microviscosity property of phospholipid. At low temperature, two acyl chains of phospholipid arrange themselves in trans form which their motion is restricted and the molecule is in ordered or solid or gel phase. When temperature increases, orientation of two acyl chains changes their conformation from trans form to gauche and trans form, as a consequence, the motion of acyl chains increase and phospholipid molecule is in disordered or fluid or liquid-crystalline phase. The temperature at which phase changed is called phase transition temperature (T_c) [New, 1990] and each phospholipid has different T_c, depending on head group, length and unsaturation bond property of their acyl chain. Egg yolk phosphatidylcholine has a T_c from -15 ºC to -7 ºC whereas synthetic DPPC is 41 ºC. The difference in T_c of both phospholipids might play an important role on OH stretching band. At the experimental temperature (about 25 ºC), EPC was in the liquid-crystalline phase that exhibited free movement of acyl chains. The fluid environment might facilitate hydrogen-bond formation in EPC reversed micelle more than in DPPC reversed micelle which their acyl chains were in ordered state. Diazepam molecules faced more difficult penetration through rigid gel phase of DPPC in interaction with bonded water, as could be seen by

slightly increase in peak intensity and area of OH stretching band upon increasing concentration of diazepam (Table 2).

Lorazepam is more polar than diazepam, thus its solubility in chloroform is low. Then the drug could be prepared and used in experiment up to 4 mM. At the same drug concentration (4 mM), the addition of lorazepam resulted in lower frequency of OH stretching band but lesser than diazepam (Table 3). Upon increasing concentration of lorazepam, enhancement of intensity and area of OH stretching bands were observed clearly in EPC reversed micelles (Table 4) with slightly increase in DPPC reversed micelles and the effect was pronounced in 15:90 mM EPC reversed micelles. The explanation might be as same as earlier described in diazepam.

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Table 1 Effect of diazepam on OH stretching frequency of reversed micelles at different molar ratio of phospholipid:D_2O. Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	3391.57±0.42	3400.20±0.08	3391.23±0.21	3391.68±0.09
4	3380.47±0.65	3391.97±0.26	3378.83±0.23	3382.89±0.06
8	3379.20±2.67	3383.86±0.18	3375.38±0.47	3382.63±0.70
12	3376.82±3.70	3384.28±0.86	3374.53±0.15	3381.45±1.21
16	3374.42±0.21	3383.42±0.27	3374.48±0.03	3382.68±0.01

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Table 2 Effect of diazepam on OH stretching intensity and area of reversed micelles at different molar ratio of phospholipid:D_2O.

Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Intensity±S.D.				Area±S.D.			
	DPPC:D_2O		EPC:D_2O		DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	0.162±0.004	0.229±0.006	0.133±0.004	0.196±0.004	45.743±1.102	64.992±1.633	36.298±1.127	53.961±1.293
4	0.168±0.004	0.232±0.006	0.152±0.002	0.210±0.003	47.238±1.145	64.950±2.922	41.633±0.599	58.793±0.903
8	0.169±0.003	0.234±0.005	0.154±0.004	0.224±0.002	46.517±0.771	65.340±1.298	41.721±1.484	62.410±0.982
12	0.170±0.002	0.233±0.006	0.160±0.004	0.213±0.006	46.547±0.588	64.608±1.666	43.455±1.124	58.402±1.815
16	0.174±0.003	0.230±0.009	0.162±0.005	0.211±0.004	47.147±0.809	63.295±2.575	42.453±1.345	57.776±1.346

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Table 3 Effect of lorazepam on OH stretching frequency of reversed micelles at different molar ratio of phospholipid:D_2O.

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	3391.74±0.18	3400.24±0.34	3391.57±0.52	3391.92±1.08
1	3390.85±0.13	3391.86±0.24	3385.85±1.94	3391.20±0.31
2	3387.22±0.23	3390.43±0.20	3385.97±0.26	3387.45±0.12
3	3387.15±0.15	3388.27±0.13	3386.55±0.07	3387.21±0.26
4	3387.04±0.13	3387.29±0.13	3385.77±0.27	3387.36±0.56

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Table 4 Effect of lorazepam on OH stretching intensity and area of reversed micelles at different molar ratio of phospholipid:D_2O.

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Intensity±S.D.				Area±S.D.			
	DPPC:D_2O		EPC:D_2O		DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	0.185±0.003	0.225±0.002	0.137±0.005	0.198±0.002	51.991±0.974	62.641±0.748	36.928±1.394	53.575±0.808
1	0.184±0.002	0.223±0.003	0.148±0.002	0.206±0.005	51.549±0.442	60.906±1.163	40.593±0.530	57.443±1.294
2	0.191±0.005	0.229±0.004	0.151±0.006	0.210±0.002	53.718±1.229	63.558±1.136	40.264±1.614	57.629±0.574
3	0.196±0.003	0.234±0.006	0.157±0.003	0.210±0.006	54.552±0.912	64.659±1.432	42.283±0.817	57.120±1.888
4	0.191±0.004	0.232±0.011	0.171±0.006	0.212±0.006	52.124±0.936	64.418±3.169	46.213±1.624	57.762±1.964

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OD stretching

Table 5 showed the effect of diazepam on OD stretching frequency of DPPC and EPC reversed micelle. The frequency shifted downward upon addition of diazepam with clearly observed in EPC reversed micelles. The downward frequency was from 2492 cm^{-1} to 2487 cm^{-1} and from 2493 cm^{-1} to 2492 cm^{-1} for EPC and DPPC reversed micelles (15:90 mM) while at higher D_2O concentration (phospholipid:D_2O = 15:135 mM), it happened to be from 2497 cm^{-1} to 2490 cm^{-1} and from 2497 cm^{-1} to 2491 cm^{-1} for EPC and DPPC reversed micelles, respectively. Downward movement of OD stretching frequency upon addition of diazepam also indicated hydrogen bond existence which might be formed between diazepam and free D_2O released from phospholipid by drug or diazepam formed continuous hydrogen bond with bonded water as described previously in effect of diazepam on OH stretching. As illustrated in Table 6, the intensity and area decreased as well as increasing diazepam concentration. This might be obtained by releasing D_2O molecules from their binding site in phospholipid caused by diazepam. However, amount of free D_2O might be few since no free OD stretching band was observed.

Upon increasing concentration of D_2O from 15:90 mM to 15:135 mM (phospholipid:D_2O), OD stretching frequency shifted upward from 2492 cm^{-1} to 2497 cm^{-1} and 2493 cm^{-1} to 2497 cm^{-1} for EPC and DPPC reversed micelle, respectively. The intensity and area also increase as concentration of D_2O increased. Enhancement of D_2O molecule in the core of reversed micelle, which has a limited space produced steric effect and resulted in higher energy required to vibrate OD bond and shifted OD stretching band to higher frequency. Similar effects to 15:90 mM reversed micelles were also achieved

with 15:135 mM reversed micelles upon addition of diazepam as shown in Table 6.

The downward frequency of OD stretching band according to addition of lorazepam was illustrated in Table 7. The intensity and area also decreased upon increasing concentration of lorazepam (Table 8). This result showed that lorazepam, as well as diazepam, could form hydrogen bond with D_2O.

Diazepam and lorazepam exerted their effect on phospholipid:D_2O reversed micelles by releasing bonded D_2O from their binding site as could be seen by decreasing intensity and area of OD stretching band; however, the OD stretching band of bonded D_2O also shifted downward upon increasing amount of both drugs. The result indicated hydrogen bond formation which might be between drug and released D_2O or drug formed continuous hydrogen bond with hydrogen bond donor or acceptor. Diazepam and lorazepam showed pronounced effect on EPC reversed micelles which were in fluid phase at studied temperature, comparing with DPPC reversed micelles.

At the same drug concentration (4 mM), it was observed that lorazepam decreased the intensity and area of OD stretching band more than diazepam. Since lorazepam possesses extra OH group comparing with diazepam, then it is more polar than diazepam and has more power for releasing of bonded D_2O molecules from phospholipids.

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Table 5 Effect of diazepam on OD stretching frequency of reversed micelles at different molar ratio of phospholipid:D_2O.

Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	2493.18±1.21	2497.83±2.20	2492.26±0.25	2497.92±0.09
4	2492.28±1.63	2495.59±0.27	2491.61±1.55	2494.56±0.66
8	2492.34±2.41	2495.09±0.26	2489.43±0.41	2495.58±0.29
12	2491.60±0.49	2494.21±1.68	2490.30±2.84	2492.52±1.91
16	2492.58±0.59	2491.33±2.72	2487.73±0.50	2490.88±0.91

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Table 6 Effect of diazepam on OD stretching intensity and area of reversed micelles at different molar ratio of phospholipid:D_2O.

Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Intensity±S.D.				Area±S.D.			
	DPPC:D_2O		EPC:D_2O		DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	0.098±0.002	0.148±0.006	0.095±0.003	0.164±0.004	14.983±0.448	23.831±1.125	14.788±0.506	27.753±0.729
4	0.088±0.002	0.134±0.005	0.086±0.001	0.134±0.005	12.820±0.276	21.536±0.987	12.713±0.217	21.405±1.046
8	0.089±0.005	0.133±0.007	0.080±0.003	0.134±0.006	12.982±0.897	20.972±1.371	11.322±0.639	21.269±1.076
12	0.089±0.004	0.114±0.010	0.084±0.005	0.128±0.004	12.956±0.758	17.212±1.780	12.041±1.017	19.928±0.732
16	0.084±0.011	0.099±0.008	0.084±0.005	0.106±0.007	12.024±2.073	14.497±1.542	12.192±0.996	15.790±1.311

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Table 7 Effect of lorazepam on OD stretching frequency of reversed micelles at different molar ratio of phospholipid:D_2O.

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	2495.25±1.02	2500.42±1.56	2491.89±1.78	2497.12±2.48
1	2492.79±0.48	2496.57±0.23	2491.35±2.96	2494.55±0.25
2	2494.12±1.31	2497.34±2.91	2489.51±0.79	2494.04±0.08
3	2491.78±1.33	2495.90±0.27	2490.26±0.17	2493.12±1.87
4	2491.98±2.49	2494.80±0.59	2488.62±0.19	2495.24±0.22

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Table 8 Effect of lorazepam on OD stretching intensity and area of reversed micelles at different molar ratio of phospholipid:D_2O.

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Intensity±S.D.				Area±S.D.			
	DPPC:D_2O		EPC:D_2O		DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	0.111±0.005	0.169±0.004	0.105±0.004	0.163±0.004	17.478±0.885	28.395±0.691	16.694±0.684	27.695±0.916
1	0.087±0.003	0.144±0.004	0.089±0.004	0.133±0.007	12.955±0.620	23.871±0.694	13.240±0.731	21.469±1.215
2	0.084±0.005	0.130±0.003	0.085±0.007	0.133±0.002	12.281±1.006	21.175±0.638	12.454±1.301	21.468±0.427
3	0.085±0.003	0.121±0.006	0.079±0.003	0.115±0.010	12.381±0.482	19.257±1.194	11.340±0.586	18.077±1.980
4	0.077±0.003	0.105±0.012	0.075±0.004	0.116±0.005	11.203±0.651	16.122±2.215	10.333±0.654	18.209±1.055

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P=O stretching

As illustrated in Table 9 and 10, the alteration of P=O stretching frequency was not quite obvious when diazepam and lorazepam were added, respectively. Since phosphate groups exist near internal aqueous phase (D_2O) of reversed micelles, far away from hydrophobic acyl chains, then few molecules of diazepam and lorazepam could arrive in phosphate group region due to their low solubility in D_2O. Therefore, their interaction with bonded D_2O, if occurred should be few. However, the P=O stretching frequency tended to slightly shift upward when diazepam and lorazepam are added. The upward frequency upon the addition of drugs exhibited releasing of bonded D_2O from phosphate head groups, resulting in upward shift of P=O stretching frequency. The data corresponded well to the result of OD stretching band.

Upon increasing hydration (15:135 mM of phospholipid:D_2O), the P=O stretching frequency was slightly lower than at the 15:90 mM of phospholipid:D_2O molar ratio. This indicated that P=O stretching group was more hydrated by D_2O molecule at high D_2O concentration.

In this experiment, data of P=O stretching intensity and area were not shown because there were disturbed signals originated from subtracting the absorption band of chloroform from that of reversed micelle as shown in Figure 18 and 19. Due to uncertainty of obtained results, the alteration of P=O stretching band could not be used to indicate the effect of drug on reversed micelles.

Table 9 Effect of diazepam on P=O stretching frequency of reversed micelles at different molar ratio of phospholipid:D_2O.

Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	1242.72±0.29	1240.18±2.04	1244.91±1.66	1242.45±0.15
4	1245.04±1.96	1238.60±0.08	1246.26±0.43	1242.72±0.29
8	1247.25±0.55	1238.84±0.22	1247.54±0.12	1242.75±0.19
12	1246.49±1.02	1239.00±0.22	1247.14±0.52	1242.66±0.66
16	1248.59±0.96	1240.00±1.77	1246.32±0.88	1244.41±2.07

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Table 10 Effect of lorazepam on P=O stretching frequency of reversed micelles at different molar ratio of phospholipid:D_2O.

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	1242.91±0.17	1241.43±1.93	1243.30±1.30	1242.15±0.09
1	1243.62±1.40	1238.73±0.10	1246.23±0.38	1242.56±0.25
2	1242.94±0.24	1239.54±1.62	1243.07±0.20	1242.64±0.25
3	1242.81±0.25	1241.45±2.38	1245.32±2.11	1239.86±1.90
4	1244.08±2.28	1240.07±1.97	1246.38±0.25	1242.71±0.12

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C=O stretching

A phospholipid molecule contains two ester groups that link the glycerol skeleton to two acyl chains. These two C=O stretching bands are overlapped each other and only one band is observed. The C=O stretching frequency remained constant at 1732 cm^{-1} upon addition of diazepam as demonstrated in Table 11. However, slightly reduction in intensity and area of C=O stretching band due to addition of diazepam was obtained (Table 12). Since overlapped bands can be resolved from each other by using mathematical program called deconvolution, the C=O stretching band affected by diazepam was tested and analyzed.

Wong et al, 1988 reported that the C=O stretching band of partially hydrated DPPC could be resolved into three components: the free sn-1 C=O band which was closer to phospholipid core, the free sn-2 C=O band which was closer to the polar head and the hydrogen-bonded sn-2 C=O band which was the hydrated sn-2 C=O. Figure 24(a) showed the deconvoluted C=O spectrum with three important bands at 1742 cm^{-1},1738 cm^{-1} and 1729 cm^{-1}. The positions of these bands agreed well with the assigned values of free sn-1, free sn-2 and hydrogen-bonded sn-2 C=O bands in deconvoluted spectra described by Wong et al. (1988), respectively. EPC reversed micelle also exhibited deconvoluted C=O spectrum with the same frequencies as described in DPPC.

The intensity and area ratios between the free and hydrated sn-2 C=O stretching band of EPC in chloroform were 2.179±0.118 and 3.066±0.333 whereas that of DPPC in chloroform were 1.842±0.143 and 2.241±0.237, respectively. Upon the hydration (15:90 mM and 15:135 mM of

phospholipid:D_2O), these ratios were less than that of phospholipid in chloroform which was not hydrated. It indicated that D_2O molecule hydrated and formed hydrogen bond with hydrated sn-2 C=O group of phospholipid evidently by increasing of intensity and area of hydrated sn-2 C=O band. As a consequence, reduction of intensity and area ratio were obtained (Table 13).

There was a partially overlap of normal infrared spectra of phospholipid and diazepam around 1690 cm^{-1} to 1710 cm^{-1} (Figure 22); however, it was not interfere the interested frequency as could be seen clearly from deconvoluted C=O stretching bands in Figure 24. No absorption of diazepam was observed from 1710 cm^{-1} to 1770 cm^{-1} and a flat baseline was shown. Upon addition of diazepam to reversed micelles, there was an obvious alteration of hydrated sn-2 C=O intensity. Increasing concentration of diazepam resulted in decreasing hydrated sn-2 C=O band (1729 cm^{-1}) as well as increasing free sn-2 C=O band (1738 cm^{-1}) (Figure 25). This phenomena could be obviously demonstrated by using intensity or area ratio between the free and hydrated sn-2 C=O stretching bands, presented in Table 13 and Figure 26. The intensity and area ratios increased in correlation with increasing amount of diazepam, suggesting diazepam capability in releasing bonded water from their binding site with C=O group.

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Table 11 Effect of diazepam on C=O stretching frequency of reversed micelles at different molar ratio of phospholipid:D_2O. Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	1732.99±0.03	1732.67±0.05	1733.07±0.01	1732.72±0.06
4	1732.98±0.02	1732.78±0.06	1733.08±0.03	1732.87±0.05
8	1732.88±0.05	1732.69±0.04	1732.97±0.22	1732.78±0.26
12	1732.88±0.04	1732.66±0.15	1733.06±0.12	1732.78±0.13
16	1732.82±0.02	1732.74±0.15	1733.06±0.02	1732.88±0.14

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Table 12 Effect of diazepam on C=O stretching intensity and area of reversed micelles at different molar ratio of phospholipid:D_2O.

Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Intensity±S.D.				Area±S.D.			
	DPPC:D_2O		EPC:D_2O		DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	0.588±0.009	0.600±0.008	0.570±0.009	0.605±0.005	17.349±0.267	17.875±0.248	16.774±0.262	18.095±0.166
4	0.583±0.007	0.585±0.012	0.606±0.005	0.612±0.008	16.720±0.203	17.039±0.318	17.308±0.139	17.673±0.204
8	0.568±0.005	0.577±0.005	0.599±0.005	0.611±0.004	15.903±0.153	16.394±0.137	16.778±0.297	17.233±0.239
12	0.554±0.007	0.587±0.007	0.583±0.010	0.587±0.013	15.122±0.201	16.193±0.156	15.817±0.324	16.184±0.469
16	0.550±0.007	0.581±0.011	0.581±0.019	0.582±0.017	14.561±0.168	15.490±0.367	15.282±0.542	15.503±0.393

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Table 13 Effect of diazepam on intensity and area ratio between the free and hydrated sn-2 C=O stretching band of reversed micelles at different molar ratio of phospholipid:D_2O.

Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Intensity ratio±S.D.				Area ratio±S.D.			
	DPPC:D_2O		EPC:D_2O		DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15.135 mM
0	1.465±0.015	1.715±0.014	1.816±0.048	1.650±0.046	1.722±0.091	2.279±0.044	2.370±0.083	2.299±0.139
4	2.788±0.094	2.466±0.114	3.237±0.103	2.706±0.117	3.505±0.190	3.067±0.137	4.357±0.186	3.749±0.249
8	6.599±0.235	3.613±0.174	4.703±0.656	5.095±0.426	9.460±0.281	4.738±0.304	5.822±0.125	7.800±0.113
12	7.923±0.360	6.494±0.157	6.551±0.781	7.091±0.044	11.539±0.153	9.346±0.814	6.543±0.279	10.446±0.210
16	13.727±1.321	8.867±0.442	8.268±0.334	9.237±0.711	21.638±1.321	13.787±1.306	10.354±0.667	11.206±1.812

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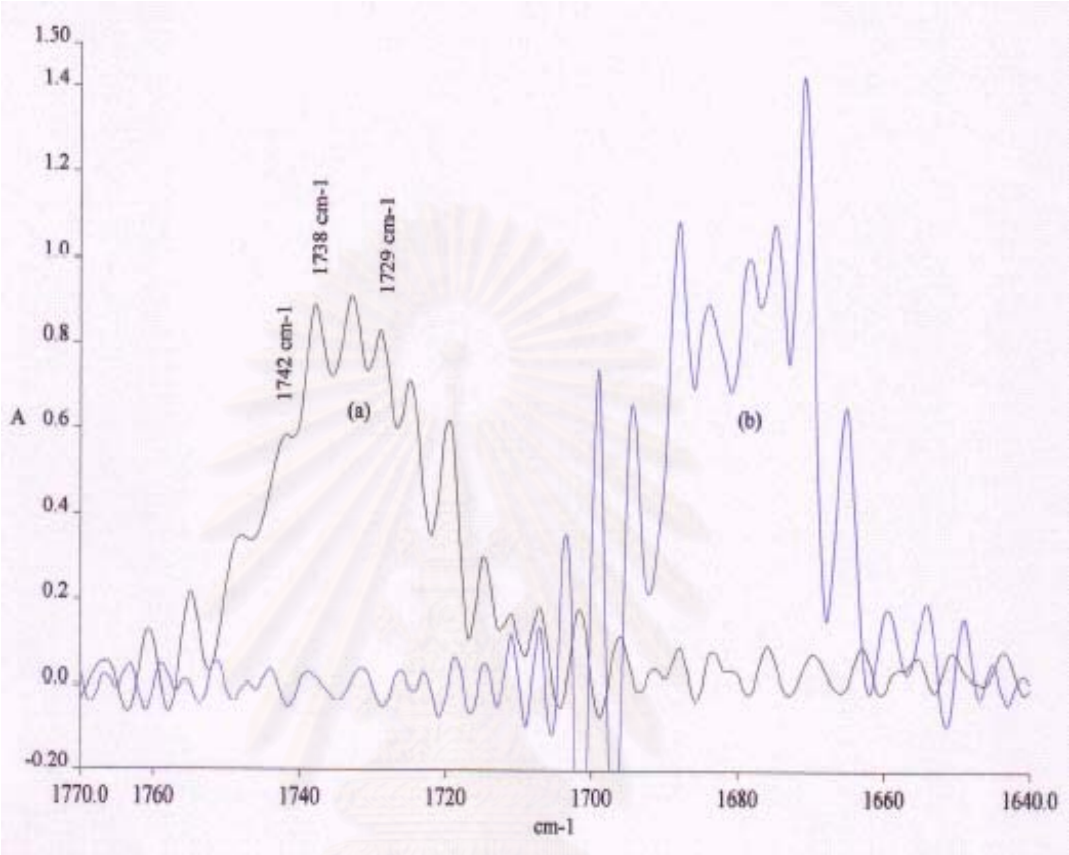

Figure 24 Deconvoluted spectra of the C=O stretching bands.
(a) DPPC-D_2O-$CHCl_3$ reversed micelle (15:90 mM).
(b) diazepam in $CHCl_3$ (16 mM).
The IR spectra were prepared by liquid seal cell technique using 0.5 mm spacer.

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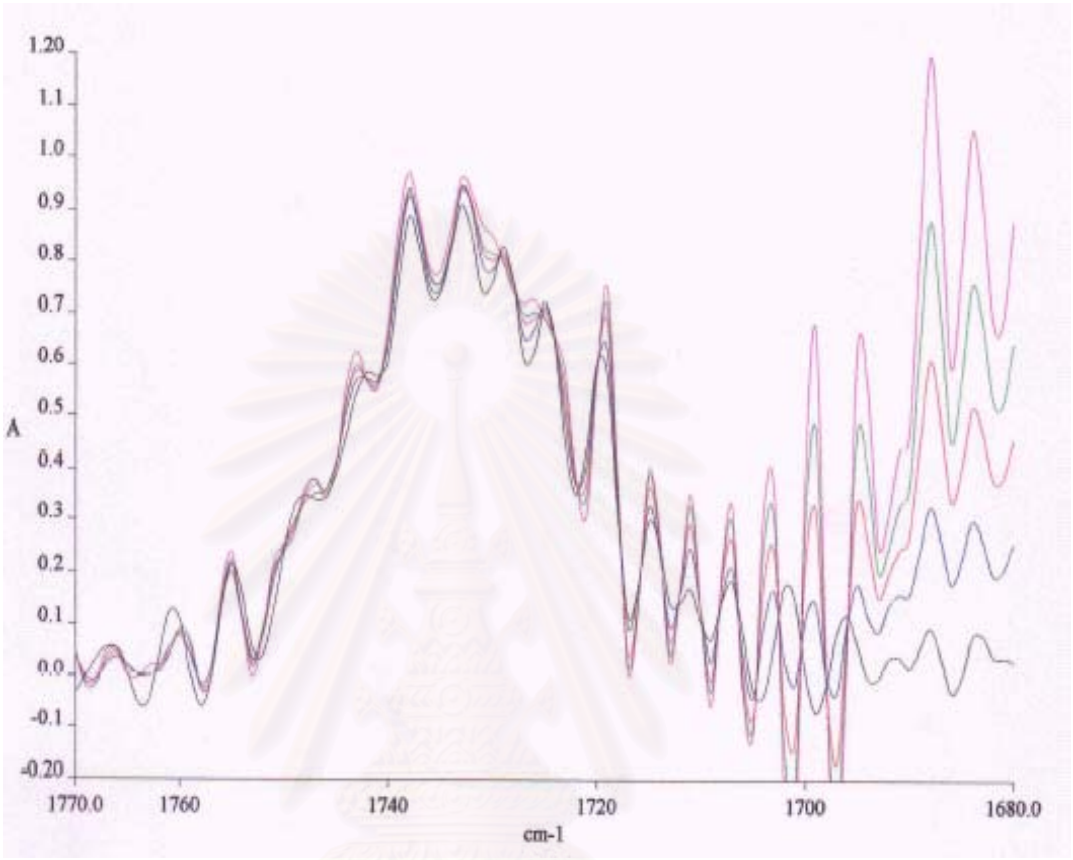

Figure 25 Effect of diazepam concentrations on deconvoluted spectra of the C=O stretching bands of DPPC-D_2O-$CHCl_3$ reversed micelle (15:90 mM).

—— 0 mM

—— 4 mM

—— 8 mM

—— 12 mM

—— 16 mM

The IR spectra were prepared by liquid seal cell technique using 0.5 mm spacer.

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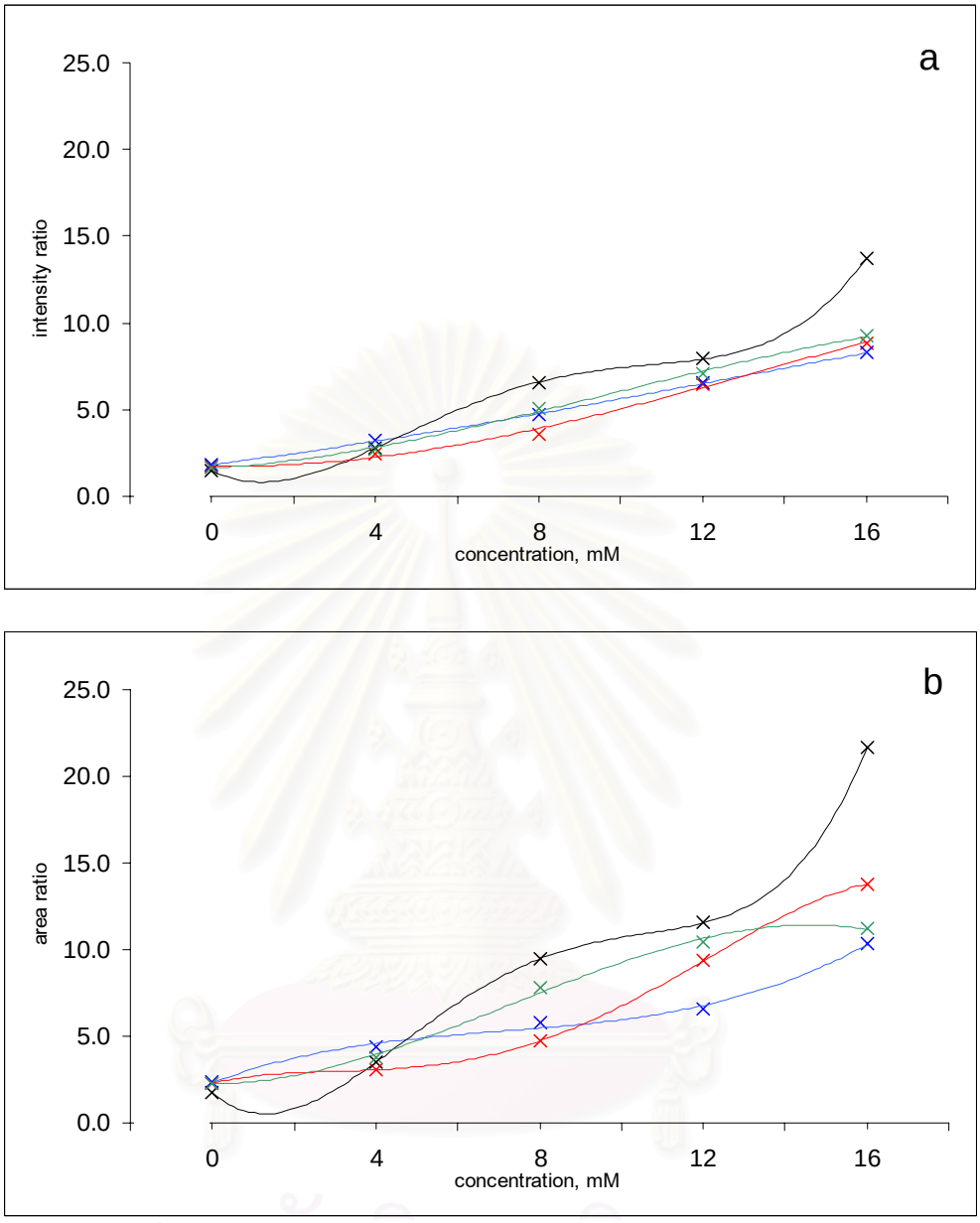

Figure 26 Effect of diazepam concentrations on the ratio between the free and hydrated sn-2 C=O stretching band of reversed micelles.
(a) intensity ratio; (b) area ratio.
— 15:90 mM of DPPC-D_2O-$CHCl_3$
— 15:135 mM of DPPC-D_2O-$CHCl_3$
— 15:90 mM of EPC-D_2O-$CHCl_3$
— 15:135 mM of EPC-D_2O-$CHCl_3$
Each point represents the mean of at least three samples±S.D., bar is omitted for clarity.

Similar results were observed with high concentration of D_2O in 15:135 mM (phospholipid:D_2O) reversed micelles (Table 12). In EPC:D_2O reversed micelles, the enhancement of intensity and area ratio of 15:90 mM and 15:135 mM (EPC:D_2O) was nearly the same (Table 12 and 13). The hydration at C=O group might be mostly obtained by traces of water (H_2O), existed in phospholipid and D_2O. Since molecule of water is smaller than of D_2O, easier penetration to C=O region should be achieved and firmly hydrogen bond formation occurred due to smaller nucleus of water, comparing with D_2O molecule. As a result of restricted penetration of D_2O molecules to narrow space around C=O group, increasing amount of D_2O in reversed micelles showed slightly effect on intensity and area of C=O stretching bands (Table 12). Therefore, diazepam exerted its activity nearly equally on releasing of bonded water molecules from hydrated sn-2 C=O group of 15:90 mM and 15:135 mM EPC reversed micelles (Table 13). However, the enhancement of intensity and area ratio of both hydrations (15:90 mM and 15:135 mM of phospholipid:D_2O) were quite similar. The most water molecules that hydrate C=O group are traces of H_2O contaminating phospholipid as shown the little decrease of this intensity and area ratio of hydrated reversed micelle compared with that of phospholipid in chloroform. In addition, the increase of D_2O might be restricted because of the limitation of space in hydrophobic region. Thus, more hydration of reversed micelle did not enhance diazepam in releasing of hydrogen-bonded water molecules from hydrated sn-2 C=O group.

Similar effect on frequency, intensity and area of C=O stretching band was observed with lorazepam as illustrated in Table 14 and 15. The C=O stretching frequency was constant at about 1733 cm^{-1} whereas the intensity and area slightly decreased when the concentration of lorazepam was increased.

The broadening region of C=O stretching bands as increasing the lorazepam concentration (Figure 23) was resolved by deconvolution as shown in Figure 27. At the maximum concentration of lorazepam used in this experiment (4mM), the rim of C=O stretching band of lorazepam was slightly overlapped with C=O stretching band of phospholipid in the region 1720 cm^{-1} to 1730 cm^{-1} as shown in Figure 28. However, the addition of lorazepam did not clearly change the deconvoluted C=O stretching intensity (Figure 27). For more obvious results, the intensity and area ratio between free and hydrated sn-2 C=O stretching band were calculated. The intensity and area ratio slightly increased upon increasing concentration of lorazepam more than 2 mM (Table 16 and Figure 29). This indicated capability of lorazepam in releasing bonded water molecules from hydrated sn-2 C=O group.

By studying deconvoluted C=O spectra, it might conclude that diazepam and lorazepam were able to release bonded water from C=O group with higher potency for diazepam at the same drug concentration (4 mM). The explanation might be the difference in solubility of these two compounds. Diazepam is more soluble in chloroform than lorazepam, hence, it could partition fairly in vicinity of glycerol backbone and provided stronger effect on withdrawing of bonded water from C=O group.

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Table 14 Effect of lorazepam on C=O stretching frequency of reversed micelles at different molar ratio of phospholipid:D_2O.

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	1732.93±0.04	1732.77±0.03	1733.08±0.13	1732.74±0.15
1	1732.92±0.03	1732.75±0.28	1733.05±0.07	1732.60±0.27
2	1732.88±0.01	1732.73±0.03	1733.06±0.03	1732.66±0.02
3	1732.76±0.03	1732.71±0.06	1732.93±0.01	1732.82±0.24
4	1732.71±0.02	1732.67±0.04	1732.92±0.13	1732.69±0.37

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Table 15 Effect of lorazepam on C=O stretching intensity and area of reversed micelles at different molar ratio of phospholipid:D_2O.

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Intensity±S.D.				Area±S.D.			
	DPPC:D_2O		EPC:D_2O		DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	0.599±0.008	0.564±0.007	0.611±0.006	0.593±0.015	17.683±0.216	16.915±0.206	17.991±0.274	17.682±0.482
1	0.589±0.007	0.573±0.001	0.610±0.005	0.585±0.009	17.212±0.177	16.905±0.280	17.562±0.147	17.202±0.234
2	0.578±0.005	0.556±0.009	0.608±0.014	0.575±0.008	16.757±0.124	16.239±0.267	17.487±0.384	16.726±0.220
3	0.570±0.008	0.560±0.011	0.592±0.007	0.577±0.018	16.352±0.221	16.182±0.313	16.867±0.196	16.481±0.732
4	0.554±0.011	0.557±0.028	0.604±0.008	0.559±0.016	15.737±0.292	15.933±0.771	16.997±0.208	15.840±0.494

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Table 16 Effect of lorazepam on intensity and area ratio between the free and hydrated sn-2 C=O stretching band of reversed micelles at different molar ratio of phospholipid:D_2O.

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Intensity ratio±S.D.				Area ratio±S.D.			
	DPPC:D_2O		EPC:D_2O		DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	1.782±0.035	1.472±0.047	1.722±0.092	2.282±0.288	2.228±0.072	1.641±0.082	2.499±0.288	2.487±0.389
1	1.702±0.038	1.415±0.020	1.823±0.068	2.223±0.100	2.251±0.095	1.632±0.019	2.225±0.107	3.362±0.254
2	1.737±0.031	1.511±0.071	1.505±0.100	2.257±0.055	2.288±0.069	1.820±0.106	1.932±0.125	3.226±0.077
3	2.376±0.055	1.977±0.047	2.559±0.081	1.775±0.034	3.478±0.134	2.609±0.049	3.996±0.133	2.351±0.242
4	2.380±0.152	2.117±0.115	2.074±0.053	1.810±0.167	3.725±0.161	2.943±0.108	3.441±0.181	2.688±0.352

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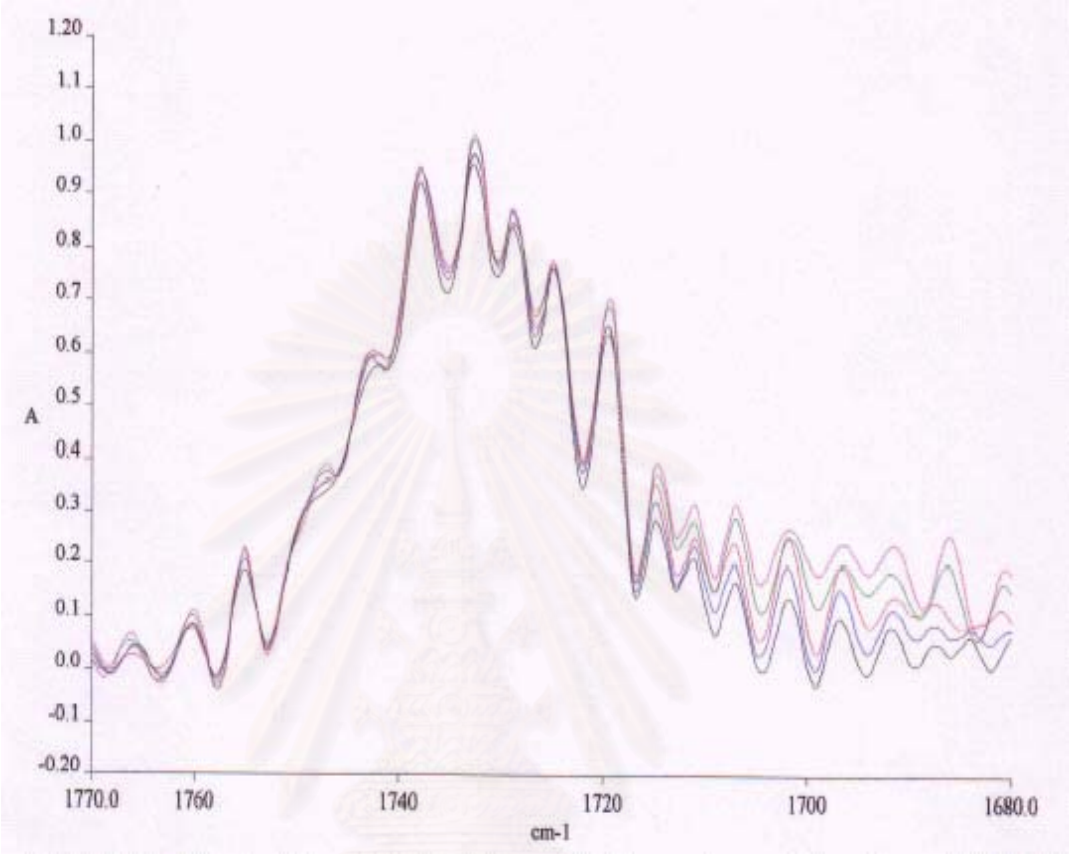

Figure 27 Effect of lorazepam concentrations on deconvoluted spectra of the C=O stretching bands of DPPC-D_2O-$CHCl_3$ reversed micelle (15:90 mM).

—— 0 mM

—— 1 mM

—— 2 mM

—— 3 mM

—— 4 mM

The IR spectra were prepared by liquid seal cell technique using 0.5 mm spacer.

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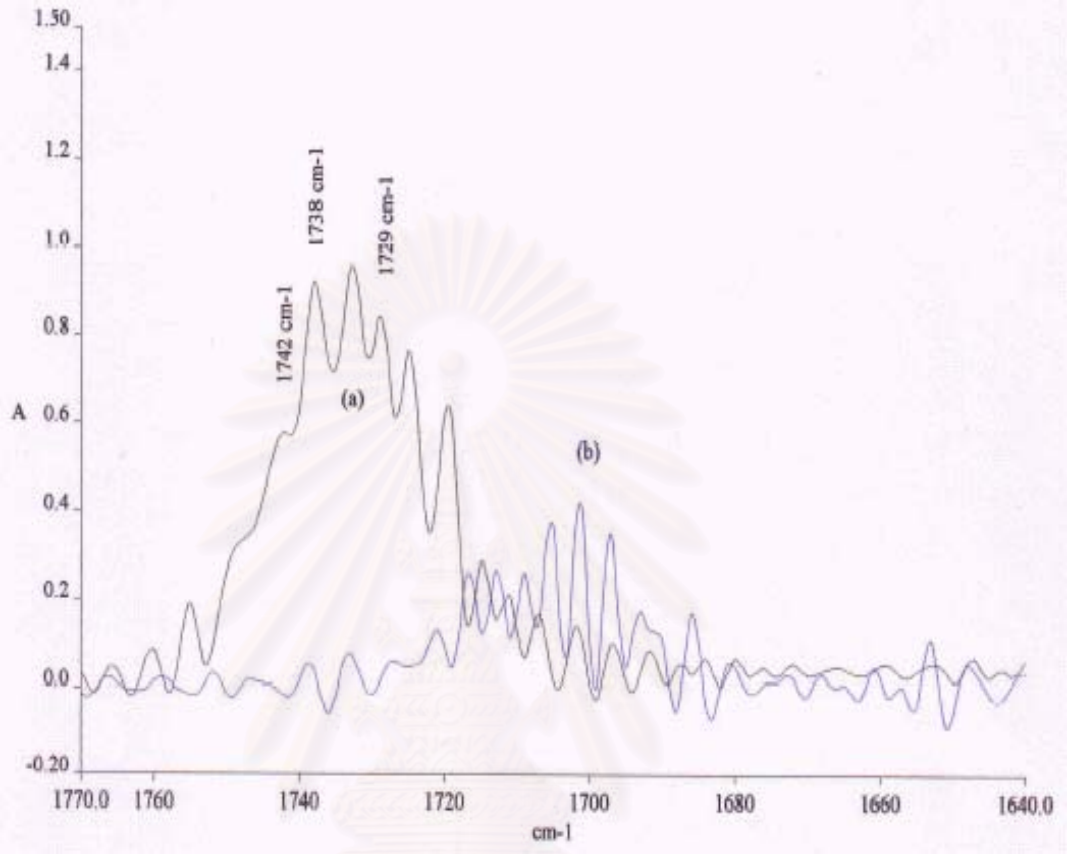

Figure 28 Deconvoluted spectra of the C=O stretching bands.
(a) DPPC-D_2O-$CHCl_3$ reversed micelle (15:90 mM).
(b) lorazepam in $CHCl_3$ (4 mM).
The IR spectra were prepared by liquid seal cell technique using 0.5 mm spacer.

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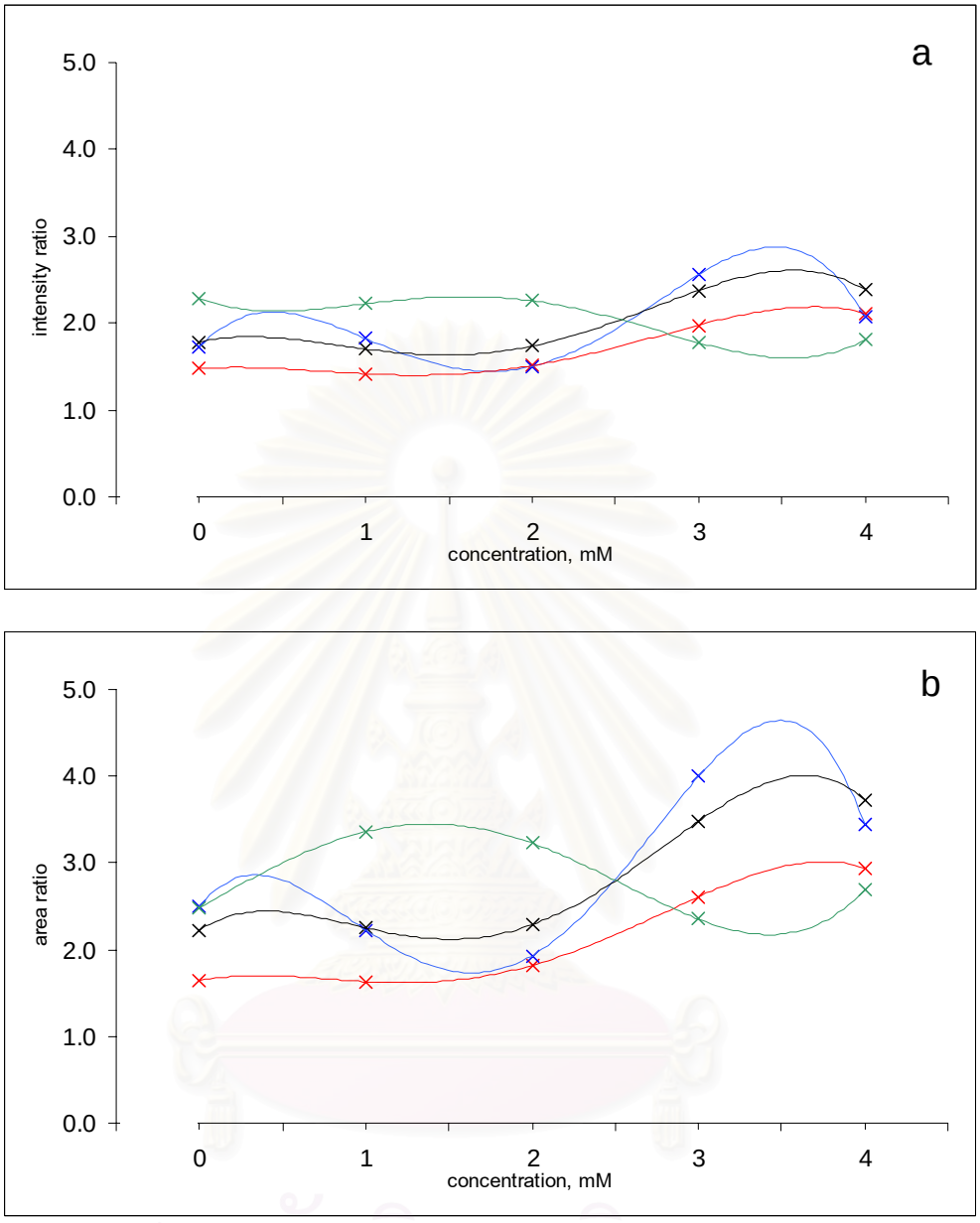

Figure 29 Effect of lorazepam concentrations on the ratio between the free and hydrated sn-2 C=O stretching band of reversed micelles.
(a) intensity ratio; (b) area ratio.
— 15:90 mM of DPPC-D_2O-$CHCl_3$
— 15:135 mM of DPPC-D_2O-$CHCl_3$
— 15:90 mM of EPC-D_2O-$CHCl_3$
— 15:135 mM of EPC-D_2O-$CHCl_3$
Each point represents the mean of at least three samples±S.D., bar is omitted for clarity.

$N^+(CH_3)_3$ stretching

Diazepam and lorazepam provided no effects on frequency, intensity and area of $N^+(CH_3)_3$ stretching band as illustrated in Table 17-20. The $N^+(CH_3)_3$ stretching frequency remained constant at about 969 cm^{-1} upon addition either diazepam and lorazepam. The choline head cannot form hydrogen bond because it lacks protons to donate and the positive charge prevents accepting proton. The hydration of the choline head is weak because the charge is covered by the three hydrophobic CH_3 groups [Ueda, 1994].

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Table 17 Effect of diazepam on $N^{+}(CH_3)_3$ stretching frequency of reversed micelles at different molar ratio of phospholipid:D_2O. Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	969.18±0.01	969.95±0.06	969.18±0.04	969.88±0.03
4	969.45±0.10	969.95±0.08	969.19±0.02	969.90±0.04
8	969.41±0.06	970.14±0.07	969.34±0.06	969.94±0.03
12	969.52±0.04	969.92±0.12	969.29±0.06	969.70±0.05
16	969.74±0.13	969.98±0.10	969.43±0.07	969.44±0.03

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Table 18 Effect of diazepam on $N^{+}(CH_3)_3$ stretching intensity and area of reversed micelles at different molar ratio of phospholipid:D_2O. Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Intensity±S.D.				Area±S.D.			
	DPPC:D_2O		EPC:D_2O		DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	0.240±0.005	0.251±0.003	0.233±0.004	0.256±0.003	5.425±0.113	5.715±0.084	5.043±0.073	5.623±0.052
4	0.244±0.003	0.249±0.004	0.251±0.003	0.264±0.003	5.663±0.077	5.711±0.196	5.631±0.066	5.955±0.076
8	0.244±0.002	0.252±0.002	0.254±0.002	0.272±0.002	5.754±0.053	5.949±0.048	5.811±0.054	6.263±0.034
12	0.243±0.003	0.260±0.001	0.256±0.004	0.266±0.006	5.843±0.053	6.285±0.012	6.033±0.095	6.219±0.106
16	0.246±0.002	0.263±0.005	0.256±0.008	0.268±0.007	6.067±0.031	6.458±0.115	6.024±0.148	6.462±0.161

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Table 19 Effect of lorazepam on $N^{+}(CH_3)_3$ stretching frequency of reversed micelles at different molar ratio of phospholipid:D_2O. Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	969.76±0.02	970.09±0.14	969.39±0.03	970.30±0.17
1	969.45±0.05	969.99±0.09	969.72±0.04	970.09±0.03
2	969.35±0.02	969.87±0.05	969.15±0.04	969.89±0.02
3	969.76±0.04	969.81±0.20	969.19±0.04	969.63±0.10
4	969.47±0.09	969.78±0.05	969.02±0.04	969.22±0.01

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Table 20 Effect of lorazepam on $N^{+}(CH_3)_3$ stretching intensity and area of reversed micelles at different molar ratio of phospholipid:D_2O.

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Intensity±S.D.				Area±S.D.			
	DPPC:D_2O		EPC:D_2O		DPPC:D_2O		EPC:D_2O	
	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM	15:90 mM	15:135 mM
0	0.244±0.003	0.236±0.003	0.250±0.003	0.250±0.005	5.507±0.075	5.333±0.081	5.385±0.061	5.315±0.089
1	0.243±0.002	0.240±0.000	0.254±0.002	0.254±0.005	5.457±0.050	5.243±0.015	5.565±0.046	5.548±0.104
2	0.247±0.001	0.241±0.003	0.259±0.005	0.255±0.003	5.631±0.033	5.421±0.062	5.633±0.101	5.603±0.074
3	0.246±0.003	0.246±0.004	0.256±0.003	0.260±0.007	5.564±0.073	5.560±0.114	5.605±0.060	5.736±0.167
4	0.242±0.004	0.251±0.010	0.268±0.004	0.259±0.006	5.493±0.074	5.758±0.206	5.939±0.092	5.808±0.141

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Effect of Benzodiazepine Drugs on Liposomes

Large multilamellar vesicles (MLV) and small unilamellar vesicles (SUV) were prepared as the representation of living cells in various sizes and the vesicles were visualized by electron microscopy as illustrated in Figure 30 and 31, respectively. These liposomes had sizes corresponding to that reported by New et al. (1990).

Figure 32(a) and (b) showed the ATR-IR spectra of DPPC MLV and SUV as a thin film on ZnSe trough plate, respectively. The frequencies of the major functional groups of DPPC liposome are:

	CH_2 antisymmetric stretching band of acyl chains at	2918 cm^{-1}
	CH_2 symmetric stretching band of acyl chains at	2850 cm^{-1}
	OH stretching band of traces of bonded water at	3370 cm^{-1}
	OD stretching band of bonded D_2O at	2491 cm^{-1}
	C=O stretching band of ester group at	1736 cm^{-1}
	P=O stretching band of phosphate moiety at	1240 cm^{-1}
and	$N^+(CH_3)_3$ stretching band of choline head at	969 cm^{-1}

In case of EPC MLV and SUV, the OH stretching, OD stretching and P=O stretching band were at 3377, 2495 and 1235 cm^{-1}, respectively (Figure 33). The lower frequency of P=O stretching band indicated that EPC liposome was more hydrated than that of DPPC liposome. Since, EPC liposomal membrane were in fluid phase at studied temperature (about 25 ºC), penetration of molecules through hydrophilic head group region of EPC liposomes was easier than in DPPC liposomes which were in solid phase. These assignments agreed

well with the corresponding bands in the phospholipid spectrum reported by Casal et al. (1984).

In this experiment, the concentrations of diazepam that could be incorporated in liposomal membrane were 0.2, 0.5, 0.7 and 1.0 mM whereas those of lorazepam were 0.5, 1.0, 1.5 and 2.0 mM. Incorporation of drugs more than that described resulted in aggregation of liposomes. As same as reversed micelle (Figure 22 and 23), the C=O stretching band of phospholipid was overlapped with C=O stretching band of drug as shown in Figure 34 and 35 of diazepam and lorazepam, respectively.

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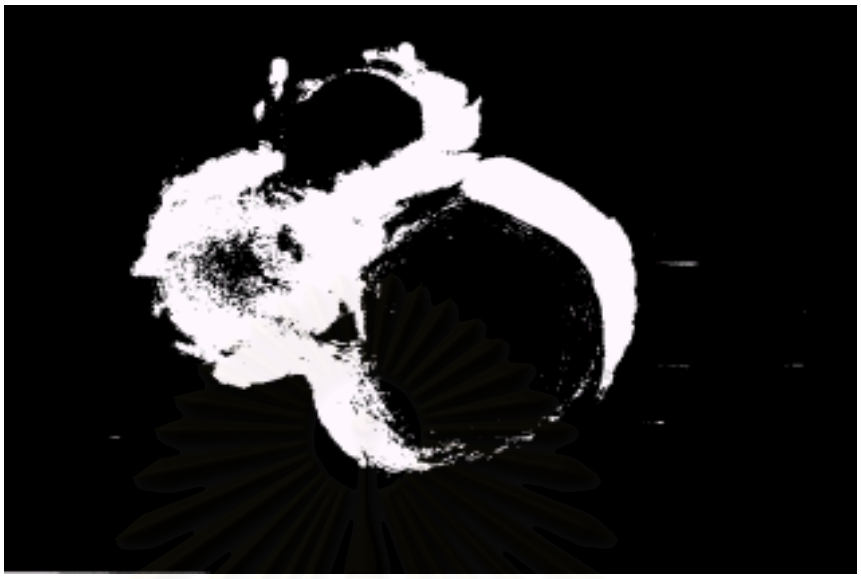

Figure 30 Electron micrograph of EPC multilamellar vesicles.
(scale : 150 mm = 1 μm)

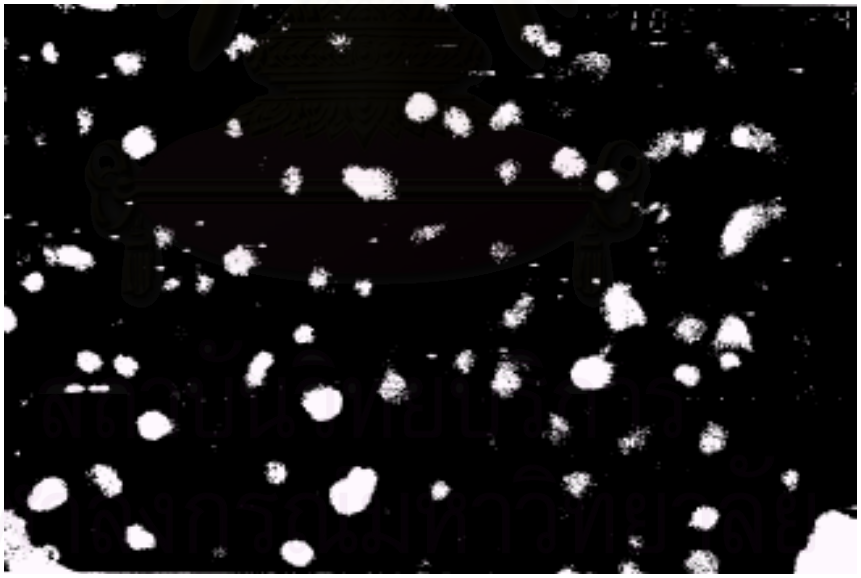

Figure 31 Electron micrograph of EPC small unilamellar vesicles.
(scale : 150 mm = 1 μm)

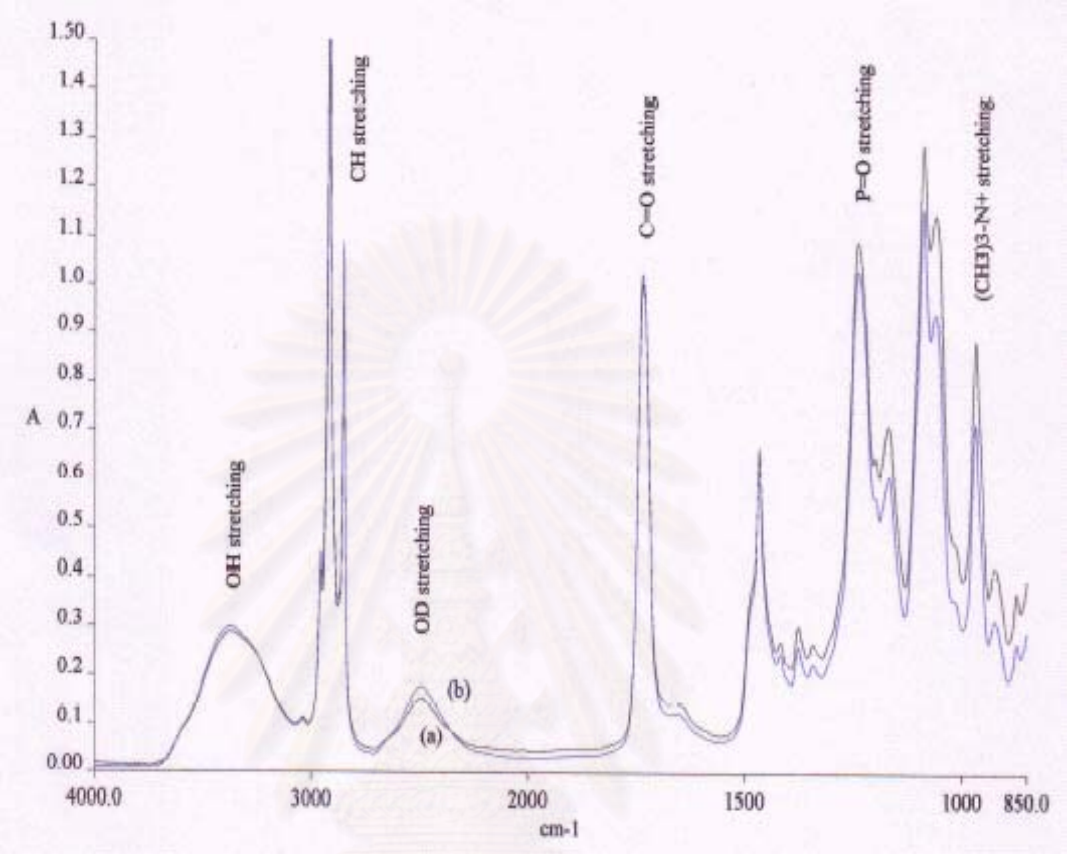

Figure 32 ATR-IR spectra of (a) DPPC MLV (___)
and (b) DPPC SUV (___).

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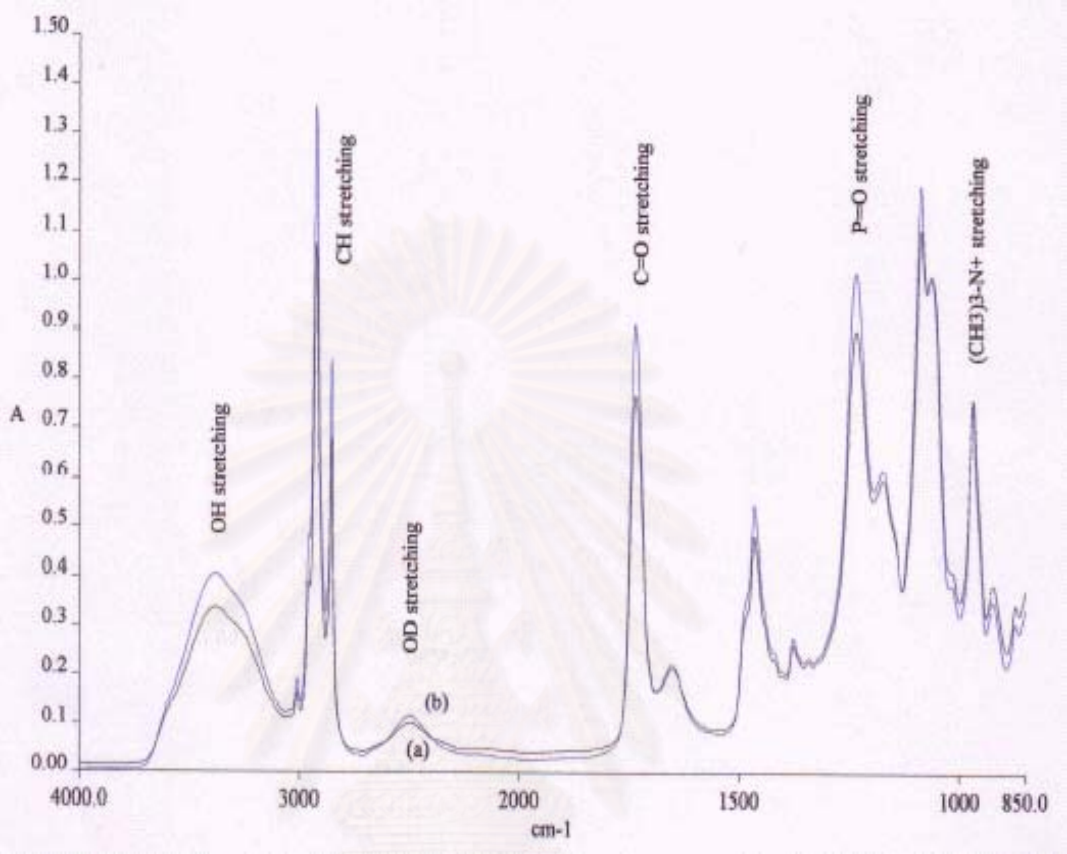

Figure 33 ATR-IR spectra of (a) EPC MLV (——) and (b) EPC SUV (——).

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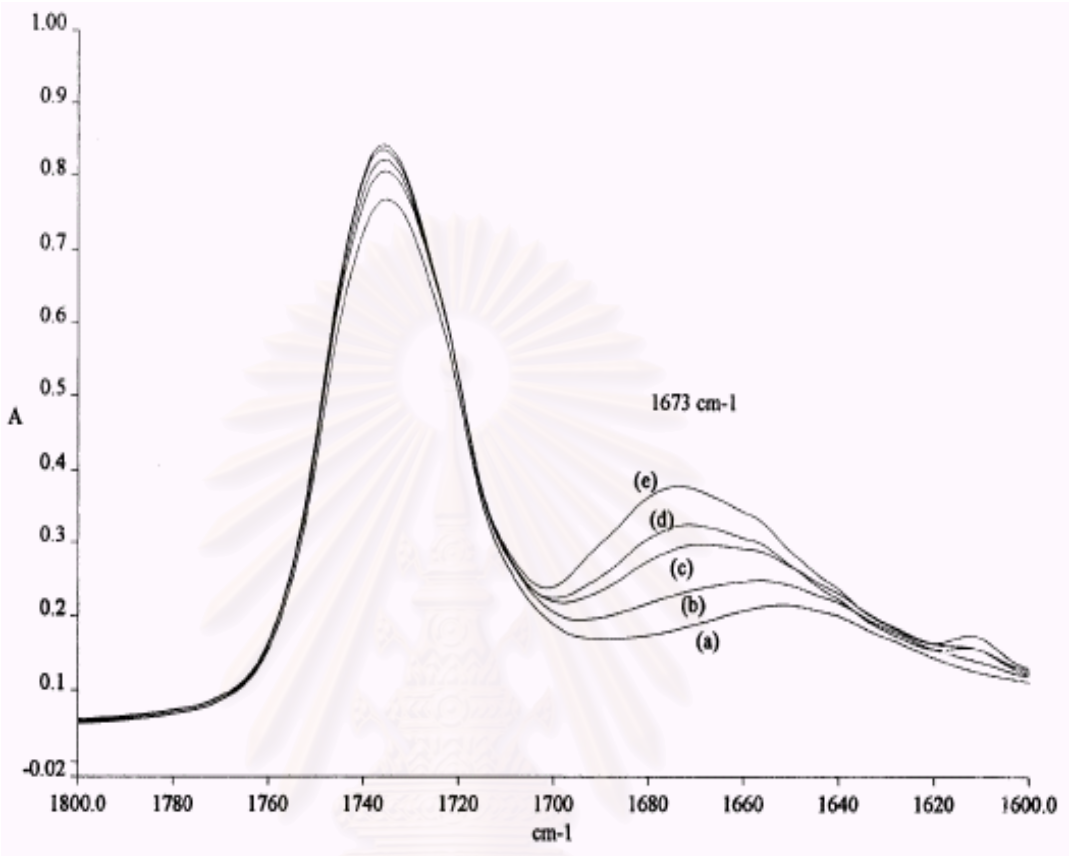

Figure 34 Effect of diazepam concentrations on the ATR-IR spectra of EPC MLV (4 mM).
(a) 0 mM; (b) 0.2 mM; (c) 0.5 mM; (d) 0.7 mM; (e) 1.0 mM.

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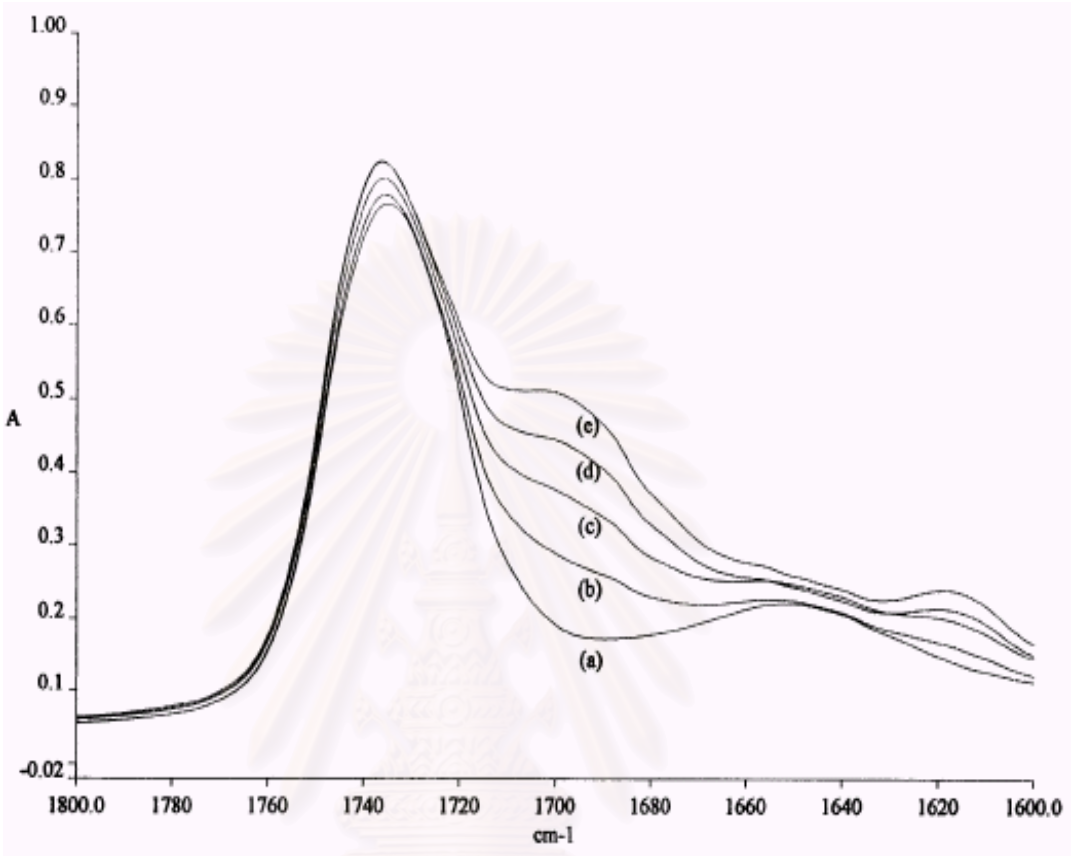

Figure 35 Effect of lorazepam concentrations on the ATR-IR spectra of EPC MLV (4 mM).
(a) 0 mM; (b) 0.5 mM; (c) 1.0 mM; (d) 1.5 mM; (e) 2.0 mM.

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OH stretching

Upon addition of diazepam to liposomal membrane, OH stretching band slightly shifted to higher frequency as illustrated in Table 21. However, enhancement in intensity and area of OH stretching band were obtained when diazepam concentration increased in all tested liposome (Table 22). Diazepam is soluble in chloroform; therefore its position in liposomal membrane should be in hydrophobic portion of liposomal membrane. Then, diazepam might provide slightly effect on bonded water in phospholipid membrane by releasing them from binding sites.

All of bonded water in liposomal membrane obtained from trace of water contaminated in phospholipid (Figure 18b) and D_2O, therefore, the quantity of bonded water in liposomal membrane should be restricted. Enhancement of intensity and area of OH stretching band upon increasing amount of diazepam might be obtained from absorption of water from atmosphere during sample preparation and measurement.

Lorazepam provided stronger effect on increasing frequency of OH stretching band (Table 23). Since lorazepam is more polar than diazepam, it should be located near hydrophilic region of liposomal membrane; thus, lorazepam might interact with bonded water by releasing them from their binding site. As a result, the frequency of bonded OH stretching shifted upward. The released water might form hydrogen bond with drug but weaker than original one since no free water band was observed. Whatever exact mechanism should be required further investigation. Variation in intensity and area of OH stretching band was obtained as shown in Table 24. This might be caused by water absorption from atmosphere as described earlier in diazepam.

OD stretching

Diazepam showed no effect on alteration of OD stretching frequency in liposomes, both MLV and SUV as illustrated in Table 25. Since large amounts of D_2O were used for preparation of liposomes, then it was difficult to control amount of D_2O left in the process of lipid film formation by evaporation. As a result, variations in intensity and area of OD stretching band were observed upon increasing amount of diazepam in liposomal membrane (Table 26).

As same as OH stretching band, OD stretching frequency shifted upward upon the addition of lorazepam (Table 27); however, variations in OD stretching intensity and area still obtained (Table 28). The possible explanation might be as same as described above for diazepam.

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Table 21 Effect of diazepam on OH stretching frequency of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC		EPC	
	MLV	SUV	MLV	SUV
0	3369.58±1.78	3370.11±0.55	3377.98±3.84	3377.69±4.87
0.2	3370.60±0.78	3371.14±0.41	3380.02±0.91	3388.51±0.28
0.5	3372.09±2.50	3371.21±0.76	3380.38±0.77	3381.36±0.54
0.7	3367.43±3.44	3372.46±0.80	3381.29±0.62	3380.55±0.33
1.0	3370.06±4.67	3372.82±1.02	3381.06±0.42	3380.76±0.62

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Table 22 Effect of diazepam on OH stretching intensity and area of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Intensity±S.D.				Area±S.D.			
	DPPC		EPC		DPPC		EPC	
	MLV	SUV	MLV	SUV	MLV	SUV	MLV	SUV
0	0.214±0.012	0.232±0.009	0.268±0.004	0.309±0.023	68.430±4.486	72.913±3.622	91.395±1.204	104.434±6.215
0.2	0.224±0.006	0.247±0.005	0.281±0.003	0.344±0.006	70.800±2.275	78.634±1.644	95.750±1.080	118.202±2.587
0.5	0.233±0.015	0.238±0.011	0.292±0.006	0.343±0.006	73.587±5.085	76.024±3.985	98.916±2.234	117.684±2.392
0.7	0.237±0.010	0.244±0.013	0.282±0.008	0.404±0.004	74.727±3.304	76.820±4.658	95.341±2.825	139.866±1.502
1.0	0.272±0.006	0.241±0.009	0.310±0.019	0.378±0.005	87.123±2.092	75.955±3.095	104.641±6.915	127.354±1.388

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Table 23 Effect of lorazepam on OH stretching frequency of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC		EPC	
	MLV	SUV	MLV	SUV
0	3369.58±1.78	3370.11±0.55	3377.98±3.84	3377.69±4.87
0.5	3372.81±1.15	3372.33±0.41	3381.17±1.21	3382.71±2.46
1.0	3376.35±2.98	3374.79±4.63	3382.17±0.77	3387.02±0.31
1.5	3378.50±2.37	3378.19±2.68	3384.06±2.02	3383.22±1.41
2.0	3380.09±0.45	3381.39±0.75	3383.05±0.48	3385.28±2.02

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Table 24 Effect of lorazepam on OH stretching intensity and area of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Intensity±S.D.				Area±S.D.			
	DPPC		EPC		DPPC		EPC	
	MLV	SUV	MLV	SUV	MLV	SUV	MLV	SUV
0	0.214±0.012	0.232±0.009	0.268±0.004	0.309±0.023	68.430±4.486	72.913±3.622	91.395±1.204	104.434±8.215
0.5	0.237±0.004	0.238±0.014	0.283±0.011	0.269±0.014	75.996±2.124	76.847±5.128	96.049±4.088	89.006±4.805
1.0	0.242±0.007	0.199±0.001	0.277±0.013	0.319±0.019	77.618±2.995	63.030±0.459	94.400±4.406	108.897±7.286
1.5	0.238±0.008	0.208±0.008	0.269±0.016	0.249±0.008	76.673±2.617	65.928±2.640	90.834±5.496	82.097±2.892
2.0	0.234±0.004	0.209±0.006	0.282±0.009	0.329±0.007	74.973±1.448	67.829±2.269	95.571±3.295	111.187±2.562

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Table 25 Effect of diazepam on OD stretching frequency of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC		EPC	
	MLV	SUV	MLV	SUV
0	2491.42±0.14	2491.20±0.15	2495.59±1.17	2495.03±0.52
0.2	2491.37±0.19	2492.00±0.21	2496.97±0.31	2497.20±1.23
0.5	2491.59±0.17	2491.79±0.12	2496.58±1.12	2497.49±0.60
0.7	2491.28±0.25	2491.64±0.50	2496.07±1.09	2499.07±0.40
1.0	2491.99±0.10	2492.04±0.22	2497.50±0.26	2497.51±0.16

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Table 26 Effect of diazepam on OD stretching intensity and area of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Intensity±S.D.				Area±S.D.			
	DPPC		EPC		DPPC		EPC	
	MLV	SUV	MLV	SUV	MLV	SUV	MLV	SUV
0	0.101±0.003	0.143±0.004	0.052±0.004	0.077±0.007	20.526±0.605	30.252±1.082	9.769±0.835	14.604±1.482
0.2	0.124±0.012	0.122±0.004	0.084±0.004	0.041±0.006	25.985±2.999	25.496±1.014	16.277±1.016	6.463±1.181
0.5	0.144±0.013	0.095±0.016	0.079±0.001	0.052±0.004	30.524±3.057	18.933±3.299	15.324±0.117	8.769±0.628
0.7	0.127±0.005	0.123±0.009	0.075±0.006	0.080±0.003	26.238±1.019	25.827±1.992	14.405±1.332	14.744±0.993
1.0	0.129±0.007	0.094±0.017	0.106±0.006	0.136±0.010	26.364±1.554	18.555±3.884	21.100±0.939	26.722±2.103

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Table 27 Effect of lorazepam on OD stretching frequency of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC		EPC	
	MLV	SUV	MLV	SUV
0	2491.42±0.14	2491.20±0.15	2495.59±1.17	2495.03±0.52
0.5	2491.81±0.66	2492.81±0.17	2497.32±0.82	2495.79±0.48
1.0	2492.56±0.21	2493.09±0.49	2498.44±0.40	2499.41±0.24
1.5	2493.40±0.48	2493.02±0.16	2498.55±0.55	2496.22±0.84
2.0	2494.01±0.92	2494.61±0.41	2499.32±0.47	2499.64±0.48

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Table 28 Effect of lorazepam on OD stretching intensity and area of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Intensity±S.D.				Area±S.D.			
	DPPC		EPC		DPPC		EPC	
	MLV	SUV	MLV	SUV	MLV	SUV	MLV	SUV
0	0.101±0.003	0.143±0.004	0.052±0.004	0.077±0.007	20.526±0.605	30.252±1.082	9.769±0.835	14.604±1.482
0.5	0.149±0.014	0.102±0.006	0.086±0.002	0.081±0.006	31.900±3.372	21.029±1.374	16.748±0.333	15.919±0.762
1.0	0.142±0.005	0.091±0.004	0.071±0.005	0.056±0.003	30.382±0.996	18.965±1.033	13.029±0.969	9.629±0.653
1.5	0.130±0.012	0.112±0.008	0.086±0.002	0.097±0.005	27.666±2.833	23.832±1.785	17.278±0.424	19.619±1.172
2.0	0.128±0.012	0.089±0.005	0.095±0.006	0.097±0.009	27.322±2.909	18.599±1.501	18.840±1.350	18.402±2.031

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P=O stretching

As illustrated in Table 29, there was no obvious change in P=O stretching frequency when diazepam was added. The P=O stretching frequencies of EPC and DPPC liposomes tended to be constant at 1235 and 1240 cm^{-1}, respectively. The P=O stretching intensity and area did not clearly show the alteration (Table 30) upon increasing amount of diazepam. Since diazepam is a slightly polar compound, its position in liposomal membrane may be far from hydrophilic region where phosphate head groups exist. As a consequence, diazepam provided slightly effect on bonded D_2O or bonded water at P=O group. Since P=O group's position was at the surface of liposomal membrane, it was easy to be hydrated by D_2O presented in large amount in liposome. As a result, if drug provided weak effect on bonded D_2O or water at P=O group, it was difficult to detect.

As illustrated in Table 31, lorazepam addition resulted in slightly decreasing frequency of P=O stretching band from 1235 cm^{-1} to 1233 cm^{-1} and 1240 cm^{-1} to 1237 cm^{-1} for EPC and DPPC liposome, respectively. However, P=O stretching intensity and area did not show any obvious change (Table 32). Since lorazepam is more polar than diazepam, its position may be close to hydrophilic head group and able to replace bonded D_2O and bonded H_2O at P=O group. The hydrogen bond formed between lorazepam and P=O might be slightly stronger than D_2O or water because more downward P=O stretching frequency was achieved.

Table 29 Effect of diazepam on P=O stretching frequency of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC		EPC	
	MLV	SUV	MLV	SUV
0	1240.31±0.09	1240.75±0.60	1234.68±0.22	1235.48±0.51
0.2	1240.18±0.24	1240.67±0.03	1234.13±0.20	1234.52±0.22
0.5	1240.12±0.27	1241.52±0.57	1235.36±0.26	1235.31±0.17
0.7	1240.39±0.12	1241.16±0.25	1235.27±0.44	1234.22±0.06
1.0	1240.09±0.19	1241.82±0.37	1234.77±0.12	1234.95±0.40

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Table 30 Effect of diazepam on P=O stretching intensity and area of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Intensity±S.D.				Area±S.D.			
	DPPC		EPC		DPPC		EPC	
	MLV	SUV	MLV	SUV	MLV	SUV	MLV	SUV
0	0.572±0.034	0.647±0.026	0.455±0.023	0.574±0.017	22.912±1.335	26.469±0.909	18.774±0.914	23.686±0.585
0.2	0.588±0.041	0.593±0.026	0.411±0.009	0.493±0.005	23.488±1.552	24.399±1.070	16.949±0.426	20.627±0.198
0.5	0.601±0.059	0.627±0.033	0.495±0.021	0.560±0.014	24.013±2.322	25.268±1.529	20.295±0.848	23.243±0.573
0.7	0.648±0.022	0.616±0.009	0.482±0.019	0.536±0.018	25.776±0.841	24.900±0.442	19.760±0.739	22.516±0.677
1.0	0.687±0.019	0.657±0.031	0.491±0.027	0.566±0.016	27.462±0.691	26.165±1.142	20.203±1.108	23.464±0.654

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Table 31 Effect of lorazepam on P=O stretching frequency of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC		EPC	
	MLV	SUV	MLV	SUV
0	1240.31±0.09	1240.75±0.60	1234.68±0.22	1235.48±0.51
0.5	1238.67±0.26	1240.19±0.29	1234.16±0.13	1236.18±0.44
1.0	1237.76±0.17	1240.44±0.38	1233.86±0.10	1234.20±0.29
1.5	1238.01±0.39	1239.55±0.22	1233.76±0.04	1235.21±0.52
2.0	1237.69±0.58	1237.36±0.34	1233.44±0.10	1233.97±0.16

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Table 32 Effect of lorazepam on P=O stretching intensity and area of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Intensity±S.D.				Area±S.D.			
	DPPC		EPC		DPPC		EPC	
	MLV	SUV	MLV	SUV	MLV	SUV	MLV	SUV
0	0.572±0.034	0.647±0.026	0.455±0.023	0.574±0.017	22.912±1.335	26.469±0.909	18.774±0.914	23.686±0.585
0.5	0.646±0.024	0.592±0.026	0.462±0.022	0.537±0.013	26.397±0.902	24.465±1.108	18.990±0.911	22.027±0.581
1.0	0.648±0.028	0.550±0.023	0.469±0.027	0.531±0.023	26.487±1.149	22.406±0.868	19.298±1.120	22.067±0.919
1.5	0.644±0.034	0.617±0.021	0.472±0.025	0.562±0.042	26.352±1.439	25.273±0.886	19.374±1.059	23.732±0.738
2.0	0.663±0.032	0.581±0.035	0.503±0.027	0.567±0.026	27.301±1.299	24.036±1.404	20.562±1.127	23.357±1.040

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C=O stretching

The two overlapped C=O stretching bands of phospholipid are observed as a single peak at 1736 cm^{-1}. As shown in Table 33, the addition of diazepam concentration did not alter the C=O stretching frequency. The intensity and area were not clearly affected by the addition of diazepam (Table 34). For study in details, the C=O stretching bands were deconvoluted. Figure 36 showed the deconvoluted C=O spectrum of EPC liposome with three important bands at 1745 cm^{-1}, 1738 $cm^{-1}$and 1731 cm^{-1}. The bands were assigned as free sn-1, free sn-2 and hydrogen-bonded sn-2 C=O stretching bands, respectively [Wong, 1988]. Similar results were also obtained with DPPC liposome. The addition of diazepam reduced hydrated sn-2 C=O stretching intensity (Figure 37) with concentration dependence. The intensity and area ratio between the free and hydrated sn-2 C=O stretching band were calculated in order to clarify effect of diazepam (Table 35 and Figure 38). The effects of diazepam on the intensity and area ratios of EPC liposome were more obvious than that of DPPC liposome. These ratios of EPC liposome increased as increasing diazepam concentration whereas that of DPPC liposome was quite constant. At experimental temperature, DPPC was in gel state that their acyl chains were rigid. As a consequence, mobility of diazepam molecules was restricted in vicinity of hydrophobic region of liposomal membrane. Therefore, diazepam had slightly effect on releasing of bonded water or D_2O from C=O group. As illustrated in Table 35 and Figure 38, there was no significant changes in intensity and area ratio between free and hydrated sn-2 C=O stretching band upon increasing concentration of diazeapam. In contrast, EPC was in fluid state at studied temperature; therefore, their acyl chains were in disordered motion. This facilitated movement of diazepam to release bonded water or D_2O from C=O group. Then, increasing concentration of diazepam increased intensity

and area ratio between free and hydrated sn-2 C=O stretching band as obviously seen in Figure 38. Vesicle size of liposome also plays an important role in drug interaction. High curvature in SUV facilitated movement of diazepam molecules since phosphate head groups were well separated from each other. MLV has a large size, thus its surface curvature is low. Then, their phosphate head groups are close together and motion of trapped molecules in MLV's membrane is more restricted than that obtained from SUV. As demonstrated in Figure 38, diazepam in SUV provided its effect on intensity and area ratio at sn-2 C=O stretching band more than diazepam in MLV.

Similar effect on the frequency of C=O stretching band was observed with lorazepam as illustrated in Table 36. The C=O stretching frequency was constant at 1736 cm^{-1}. Table 37 obviously showed that the intensity and area of C=O stretching band decreased upon increasing concentration of lorazepam. Figure 39 showed the alteration of deconvoluted C=O spectra when lorazepam was added. For more obvious results, the intensity and area ratio between the free and hydrated sn-2 C=O stretching band were calculated. Increasing in intensity and area ratios were observed as in Table 38 and Figure 40. The effect of kind of phospholipids and also size of liposome upon lorazepam addition might be similar to diazepam for releasing of bonded water or D_2O from C=O groups. As demonstrated in Table 38 and Figure 40 , lorazepam in EPC liposomes exhibited stronger effect in releasing of bonded water or D_2O from C=O groups more than in DPPC liposome and DPPC SUV provided stronger effect of lorazepam than in DPPC MLV. However, EPC MLV gave stronger effect of lorazepam than EPC SUV. Since there was slightly overlap between deconvoluted spectra of C=O stretching bands of phospholipid and C=O

stretching band of lorazepam (Figure 39) which might be contributed to this unusual phenomena.

In this experiment, diazepam and lorazepam showed similar effect on the C=O group. They could release hydrogen-bonded water from hydrated sn-2 C=O group with more potency effect in lorazepam. Since lorazepam is more polar than diazepam, then its position in liposomal membrane might be closer to C=O region than diazepam. Thus, releasing of bonded water or D_2O from C=O group might be easier in lorazepam than diazepam.

$N^+(CH_3)_3$ stretching

Addition of diazepam and lorazepam to MLV and SUV provided no effect on $N^+(CH_3)_3$ stretching frequency and also on its intensity and band area as shown in Table 39 to Table 42. Although the choline group has a positive charge, it cannot form a hydrogen bond because there is no proton to donate and the positive charge prevents the acceptance of protons [Chiou, 1992 and Ueda, 1994]. From data presented, the infrared signal of $N^+(CH_3)_3$ stretching band was unresponsive to benzodiazepines.

From overall studied data, it might be concluded that main activity of diazepam and lorazepam was to release hydrogen bond between water and sn-2 C=O group of phospholipid. The activity depended on physicochemical property of drug, type of lipid and also size of liposome. Lorazepam provided stronger effect than diazepam due to possessing more hydrogen donor and acceptor in molecule.

Table 33 Effect of diazepam on C=O stretching frequency of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC		EPC	
	MLV	SUV	MLV	SUV
0	1735.58±0.04	1736.20±0.13	1735.34±0.13	1736.05±0.15
0.2	1735.57±0.15	1736.43±0.04	1735.35±0.07	1735.88±0.04
0.5	1735.67±0.08	1736.40±0.10	1735.80±0.14	1736.04±0.07
0.7	1735.62±0.04	1736.45±0.04	1735.85±0.14	1735.98±0.02
1.0	1735.69±0.03	1736.32±0.04	1735.92±0.05	1736.02±0.19

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Table 34 Effect of diazepam on C=O stretching intensity and area of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Intensity±S.D.				Area±S.D.			
	DPPC		EPC		DPPC		EPC	
	MLV	SUV	MLV	SUV	MLV	SUV	MLV	SUV
0	0.816±0.045	0.938±0.033	0.626±0.027	0.790±0.020	24.063±1.380	27.250±0.827	19.528±1.026	24.183±0.533
0.2	0.829±0.051	0.884±0.030	0.560±0.012	0.688±0.004	24.235±1.414	25.360±0.863	17.414±0.399	21.133±0.158
0.5	0.843±0.070	0.917±0.040	0.676±0.022	0.762±0.015	24.354±1.988	25.891±1.250	20.373±0.645	22.933±0.419
0.7	0.891±0.028	0.906±0.011	0.654±0.026	0.726±0.019	25.440±0.737	25.478±0.349	19.515±0.709	21.855±0.668
1.0	0.938±0.023	0.925±0.032	0.654±0.030	0.749±0.013	26.530±0.609	26.600±0.844	19.390±0.974	22.344±0.350

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Table 35 Effect of diazepam on the intensity and area ratio between the free and hydrated sn-2 C=O stretching band of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Intensity ratio±S.D.				Area ratio±S.D.			
	DPPC		EPC		DPPC		EPC	
	MLV	SUV	MLV	SUV	MLV	SUV	MLV	SUV
0	1.411±0.060	1.731±0.054	1.653±0.089	1.947±0.050	1.671±0.061	2.097±0.064	1.841±0.097	2.285±0.068
0.2	1.506±0.034	1.880±0.089	1.933±0.075	2.109±0.078	1.763±0.033	2.221±0.101	2.154±0.083	2.423±0.093
0.5	1.549±0.084	1.844±0.061	2.277±0.025	2.376±0.060	1.826±0.097	2.189±0.067	2.600±0.046	2.766±0.065
0.7	1.434±0.045	1.791±0.026	2.645±0.102	2.718±0.096	1.681±0.051	2.102±0.030	2.983±0.106	3.224±0.156
1.0	1.391±0.029	1.758±0.072	3.316±0.075	2.791±0.162	1.609±0.045	2.061±0.101	3.623±0.029	3.252±0.229

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Table 36 Effect of lorazepam on C=O stretching frequency of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC		EPC	
	MLV	SUV	MLV	SUV
0	1735.58±0.04	1736.20±0.13	1735.34±0.13	1736.05±0.15
0.5	1735.66±0.08	1736.42±0.04	1735.66±0.04	1736.26±0.09
1.0	1735.86±0.02	1736.60±0.02	1735.96±0.04	1736.37±0.03
1.5	1736.20±0.11	1736.54±0.04	1736.21±0.06	1736.67±0.03
2.0	1736.22±0.18	1736.56±0.05	1736.40±0.01	1736.56±0.05

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Table 37 Effect of lorazepam on C=O stretching intensity and area of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Intensity±S.D.				Area±S.D.			
	DPPC		EPC		DPPC		EPC	
	MLV	SUV	MLV	SUV	MLV	SUV	MLV	SUV
0	0.816±0.045	0.938±0.033	0.626±0.027	0.790±0.020	24.063±1.380	27.250±0.827	19.528±1.026	24.183±0.533
0.5	0.846±0.031	0.841±0.033	0.586±0.026	0.700±0.013	24.587±0.963	23.966±0.910	17.758±0.784	20.752±0.438
1.0	0.805±0.037	0.759±0.028	0.564±0.027	0.659±0.023	22.796±1.031	21.231±0.741	16.566±0.722	19.429±0.598
1.5	0.770±0.031	0.782±0.030	0.546±0.029	0.693±0.017	21.191±0.884	21.571±0.836	15.605±0.812	19.677±0.494
2.0	0.767±0.060	0.707±0.033	0.547±0.024	0.639±0.022	20.954±2.044	19.288±0.896	15.265±0.665	18.062±0.601

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Table 38 Effect of lorazepam on the intensity and area ratio between the free and hydrated sn-2 C=O stretching band of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Intensity ratio±S.D.				Area ratio±S.D.			
	DPPC		EPC		DPPC		EPC	
	MLV	SUV	MLV	SUV	MLV	SUV	MLV	SUV
0	1.411±0.060	1.731±0.054	1.653±0.089	1.947±0.050	1.671±0.061	2.097±0.064	1.841±0.097	2.285±0.068
0.5	1.587±0.082	2.242±0.076	2.472±0.106	2.556±0.093	1.874±0.077	2.603±0.078	2.705±0.120	2.842±0.095
1.0	2.210±0.091	2.895±0.120	3.687±0.149	3.192±0.100	2.471±0.085	3.188±0.084	3.905±0.140	3.466±0.099
1.5	2.832±0.116	3.216±0.119	5.707±0.938	4.000±0.144	3.064±0.118	3.440±0.105	4.664±0.129	4.229±0.042
2.0	3.443±0.106	3.864±0.116	9.790±0.670	5.178±0.119	3.592±0.049	4.025±0.098	5.373±0.142	5.127±0.115

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Table 39 Effect of diazepam on $N^{+}(CH_3)_3$ stretching frequency of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC		EPC	
	MLV	SUV	MLV	SUV
0	968.72±0.02	969.25±0.06	968.95±0.07	969.18±0.08
0.2	968.67±0.03	969.49±0.05	969.01±0.03	969.48±0.05
0.5	968.87±0.06	969.30±0.04	968.98±0.12	969.22±0.04
0.7	968.82±0.01	969.46±0.03	968.96±0.06	969.46±0.04
1.0	968.93±0.02	969.25±0.10	969.04±0.05	969.34±0.05

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Table 40 Effect of diazepam on $N^{+}(CH_3)_3$ stretching intensity and area of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of diazepam (mM)	Intensity±S.D.				Area±S.D.			
	DPPC		EPC		DPPC		EPC	
	MLV	SUV	MLV	SUV	MLV	SUV	MLV	SUV
0	0.479±0.024	0.435±0.013	0.409±0.031	0.448±0.014	11.256±0.543	9.978±0.302	9.566±0.748	10.362±0.333
0.2	0.492±0.029	0.394±0.020	0.372±0.012	0.378±0.005	11.585±0.663	9.055±0.460	8.702±0.291	8.679±0.114
0.5	0.501±0.054	0.423±0.034	0.433±0.029	0.435±0.017	11.804±1.254	9.777±0.793	10.164±0.700	10.012±0.387
0.7	0.541±0.014	0.422±0.010	0.423±0.024	0.420±0.017	12.732±0.310	9.776±0.244	9.950±0.601	9.653±0.409
1.0	0.567±0.013	0.455±0.023	0.426±0.028	0.439±0.015	13.274±0.310	10.560±0.530	9.974±0.627	10.159±0.346

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Table 41 Effect of lorazepam on $N^{+}(CH_3)_3$ stretching frequency of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Frequency (cm^{-1})±S.D.			
	DPPC		EPC	
	MLV	SUV	MLV	SUV
0	968.72±0.02	969.25±0.06	968.95±0.07	969.18±0.08
0.5	968.83±0.06	969.34±0.05	968.88±0.08	969.02±0.05
1.0	968.94±0.05	969.30±0.08	968.92±0.04	969.31±0.07
1.5	969.04±0.05	969.20±0.05	968.94±0.05	969.06±0.03
2.0	968.98±0.07	969.24±0.08	968.92±0.01	969.28±0.06

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Table 42 Effect of lorazepam on $N^{+}(CH_3)_3$ stretching intensity and area of liposomes with different size (MLV and SUV).

Data represented the mean of at least three samples±S.D.

Concentration of lorazepam (mM)	Intensity±S.D.				Area±S.D.			
	DPPC		EPC		DPPC		EPC	
	MLV	SUV	MLV	SUV	MLV	SUV	MLV	SUV
0	0.479±0.024	0.435±0.013	0.409±0.031	0.448±0.014	11.256±0.543	9.978±0.302	9.566±0.748	10.362±0.333
0.5	0.537±0.028	0.398±0.021	0.414±0.026	0.401±0.012	12.514±0.657	9.133±0.485	9.655±0.631	9.198±0.281
1.0	0.526±0.013	0.359±0.015	0.415±0.022	0.401±0.019	12.228±0.263	8.232±0.348	9.646±0.516	9.137±0.425
1.5	0.479±0.035	0.420±0.011	0.407±0.019	0.427±0.016	11.017±0.816	9.650±0.263	9.449±0.429	9.800±0.366
2.0	0.493±0.024	0.402±0.029	0.433±0.026	0.421±0.022	11.332±0.571	9.208±0.671	9.992±0.561	9.690±0.505

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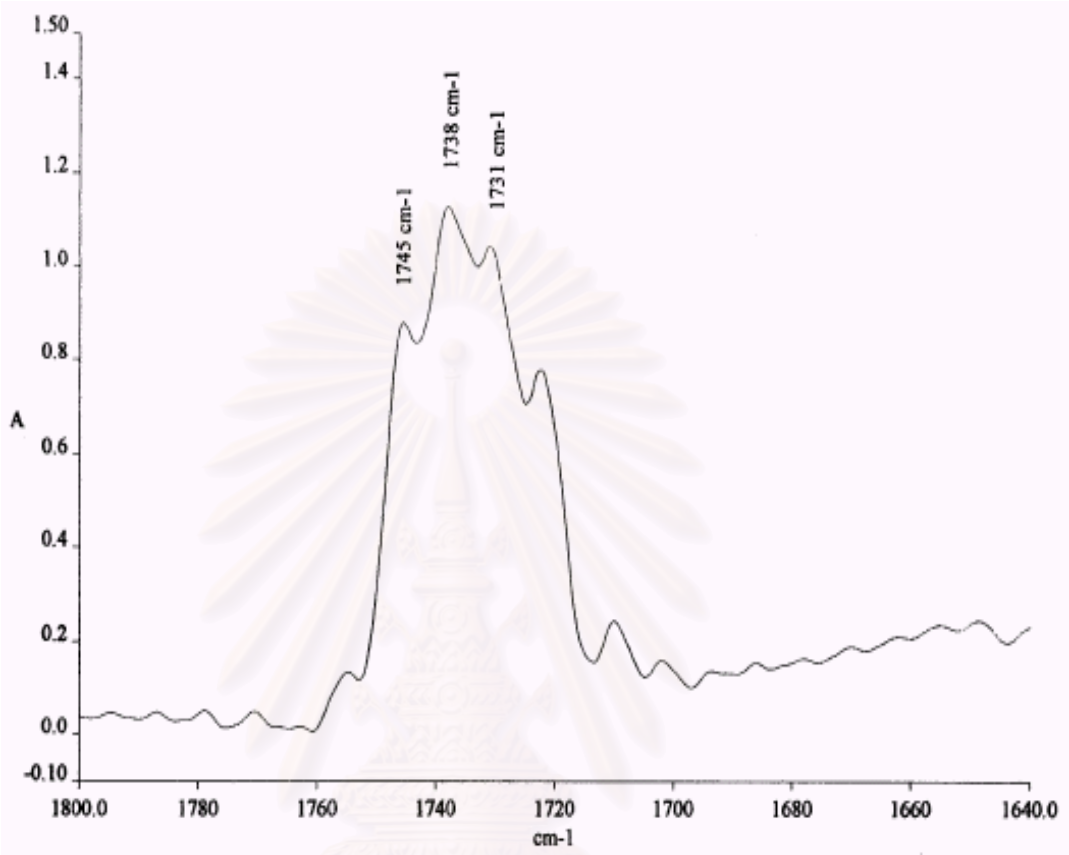

Figure 36 Deconvoluted ATR-IR spectrum of the C=O stretching band of EPC MLV (4 mM).

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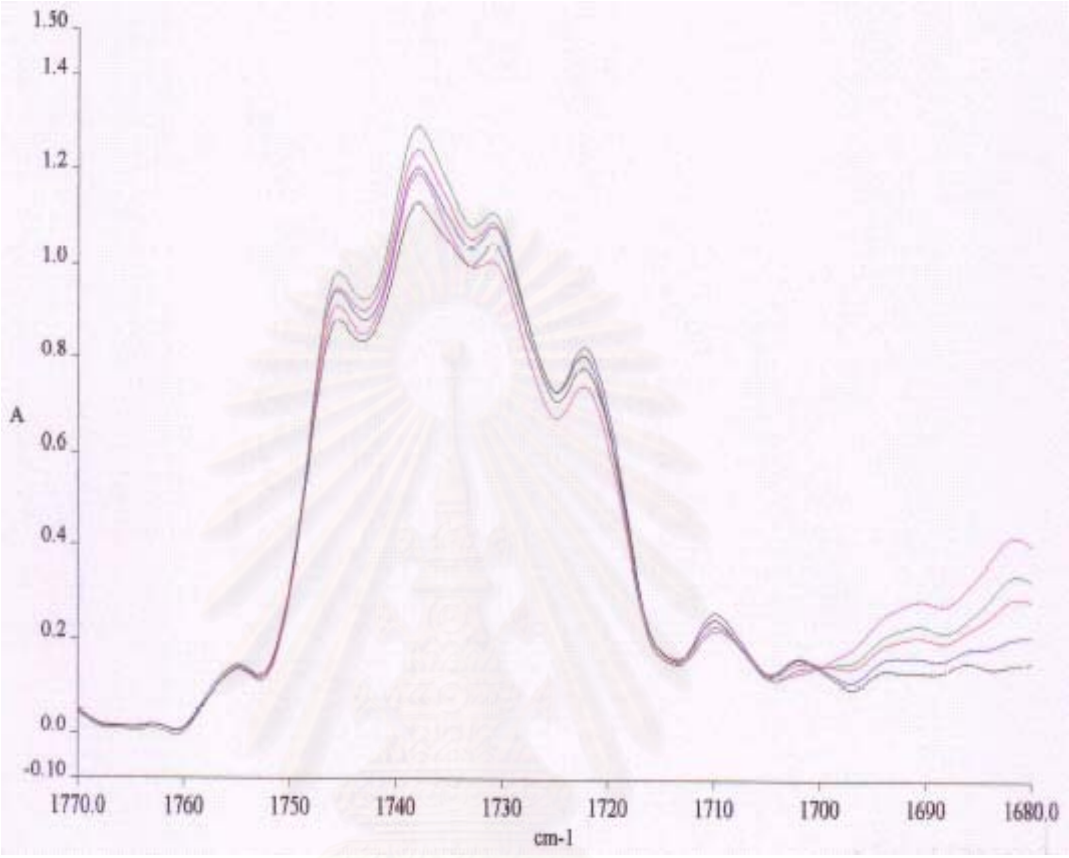

Figure 37 Effect of diazepam concentrations on deconvoluted ATR-IR spectra of the C=O stretching bands of EPC MLV (4 mM).

—— 0 mM

—— 0.2 mM

—— 0.5 mM

—— 0.7 mM

—— 1.0 mM

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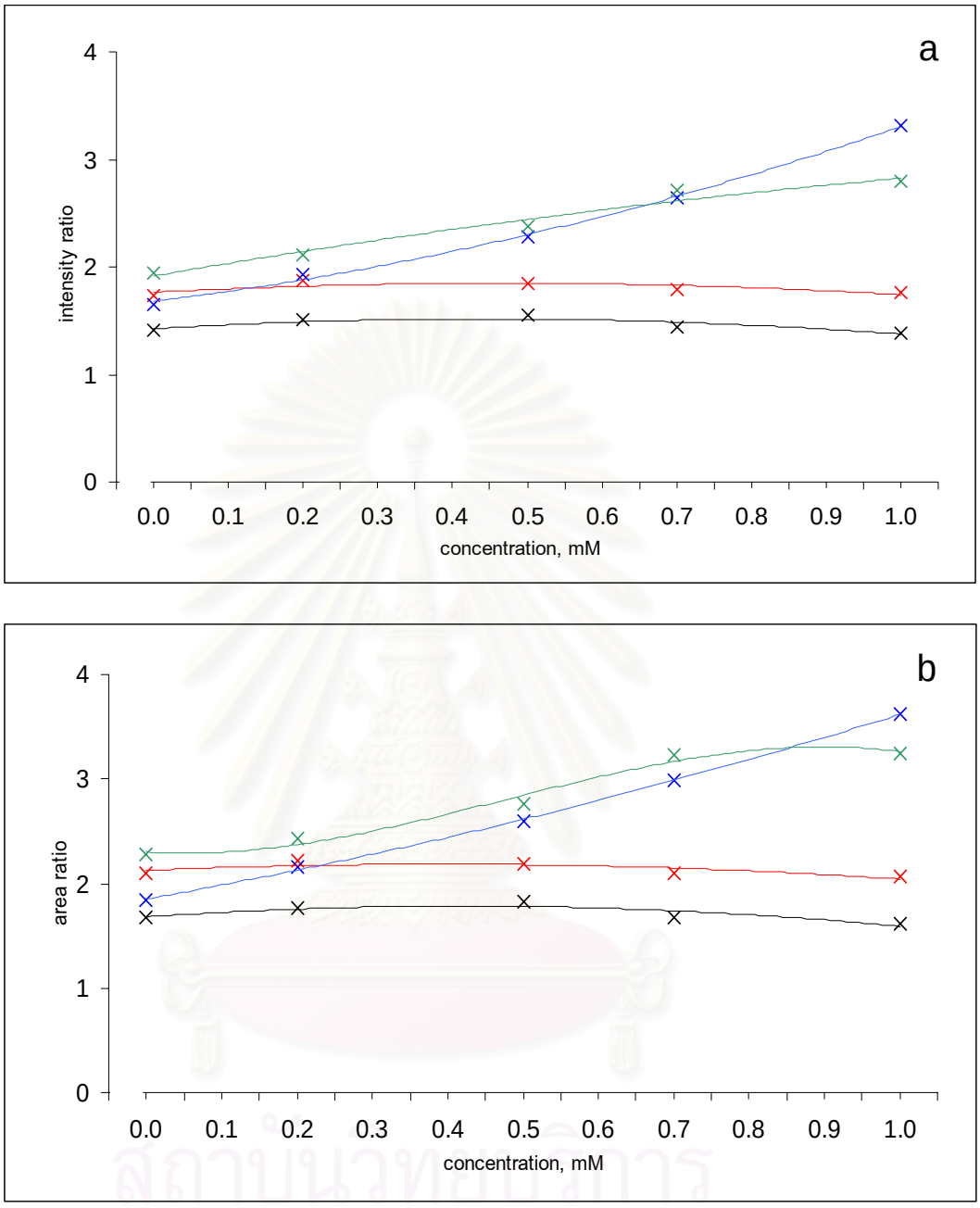

Figure 38 Effect of diazepam concentrations on the ratio between the free and hydrated sn-2 C=O stretching band of liposomes.
(a) intensity ratio; (b) area ratio.

DPPC MLV

DPPC SUV

EPC MLV

EPC SUV

Each point represents the mean of at least three samples±S.D., bar is omitted for clarity.

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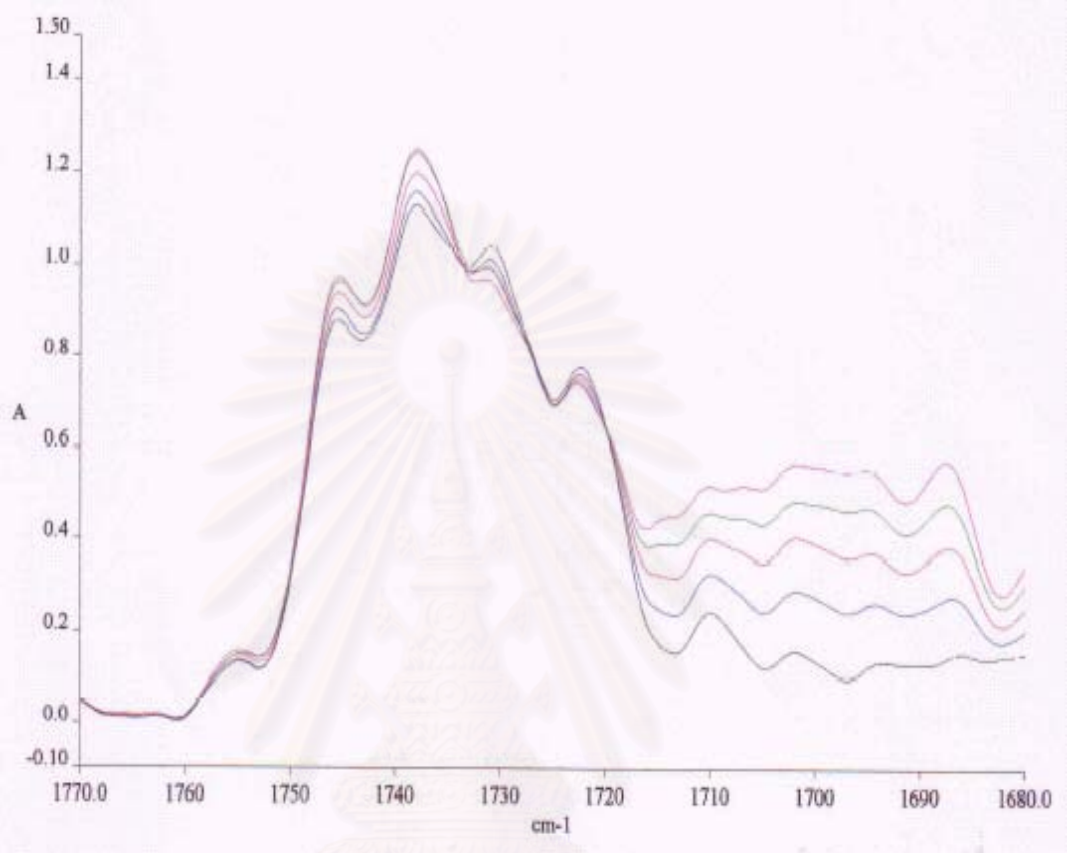

Figure 39 Effect of lorazepam concentrations on deconvoluted ATR-IR spectra of the C=O stretching bands of EPC MLV (4 mM).

—— 0 mM

—— 0.5 mM

—— 1.0 mM

—— 1.5 mM

—— 2.0 mM

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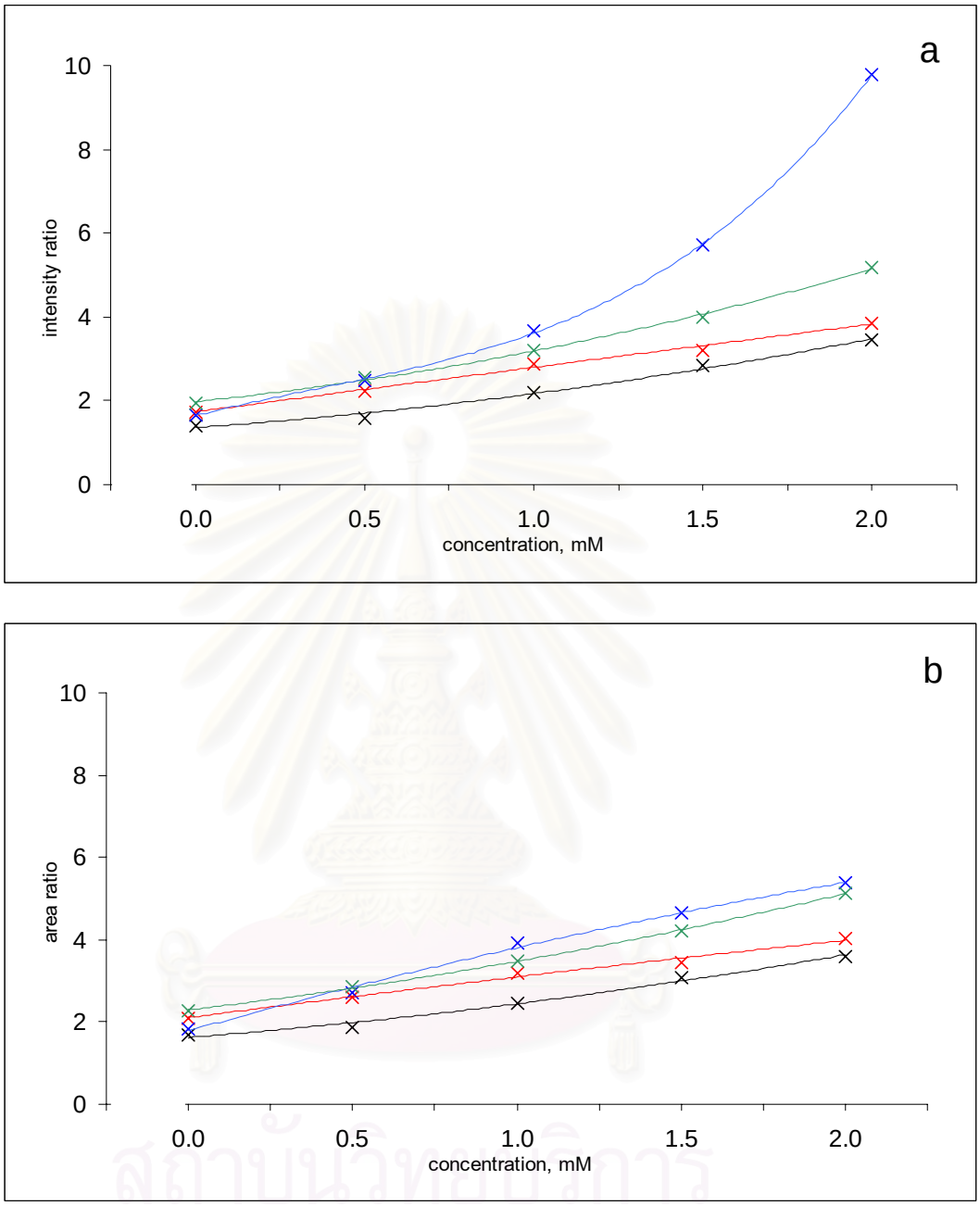

Figure 40 Effect of lorazepam concentrations on the ratio between the free and hydrated sn-2 C=O stretching band of liposomes.
(a) intensity ratio; (b) area ratio.

—— DPPC MLV

—— DPPC SUV

—— EPC MLV

—— EPC SUV

Each point represents the mean of at least three samples±S.D., bar is omitted for clarity.

CHAPTER V

CONCLUSION

Biological membrane composes of phospholipids which their molecules are divided into hydrophilic head group and hydrophobic tail. Phospholipids form a bimolecular structure hold molecules together by Van der Waal interactions between hydrocarbon chains and hydrogen bonds between the polar head groups and water. The factor that weakens these forces may disorder and fluidize the membrane structure. The previous studies showed that high pressure, alcohols and anesthetics interrupted the hydrogen bonds between water and phospholipid molecule and then released the water molecule. From these results indicated that the hydrogen bond was important to support the membrane structure and also acted as the site of action on phospholipid membrane.

Benzodiazepines, sedative-hypnotics, have the pharmacological action via GABA receptor and can fluidize the phospholipid membrane. Thus, this study investigated the hydrogen bond interaction between benzodiazepines, diazepam and lorazepam, and phospholipid membranes to examine whether it is the site of action. Infrared spectroscopic technique is selected to investigate this interaction while reversed micelle and liposome are used as model membranes.

From the result of diazepam and lorazepam on reversed micelles, they could affect the hydrogen bond between water and phospholipid. Diazepam clearly provided the effect on the sn-2 C=O group located closely to hydrophobic region. It released hydrogen bond between traces of water and sn-2 C=O group. Lorazepam provided stronger effect on bonded D_2O with a lesser effect on C=O stretching band comparing with diazepam. Solubility of drugs is the limiting factor that governs its function in studied model. Since, lorazepam is more polar than diazepam, thus its position in reversed micelle might be closer to hydrophilic head group which enriched with D_2O while diazepam existed in vicinity of hydrophobic region near C=O group. Membrane fluidity played a significant role in this investigation. EPC being more fluidize than DPPC showed the enhancement of hydrogen bond releasing between water and phospholipid.

In case of liposomes, diazepam and lorazepam could interrupt the hydrogen bond between traces of bound water and sn-2 C=O group in vicinity closely to hydrophobic region. However, the result clearly showed that lorazepam which is polar than diazepam provided stronger activity since it could be located nearer site of action.

The overall results of benzodiazepines on hydrogen bonding in phospholipid membrane could conclude that benzodiazepines mainly released hydrogen bond between water and phospholipid in vicinity closely to hydrophobic region. Drug solubility, membrane fluidity and size of vesicle were the important factors influencing the interaction.

In this study, infrared spectroscopy can be used as a method of choice for biomembrane analysis. It is sensitive to assess dynamics of each part of the phospholipid molecule. ATR-IR spectroscopic technique is suitable for study of biological membranes and can give the informative result at the water-membrane interface. Thus, infrared spectroscopic technique can be applied successfully to the study of other biological membrane researches. In addition, the reversed micelle is one of suitable models with the advantage that the behavior of water molecule is easily controlled and identified due to restricted amount of water.

For further investigation, benzodiazepines at various concentrations should be examined their action on hydrogen bonding, in vivo, in native cell membrane of healthy person and patient. The correlation between amount of drug used in therapy and their efficiency on interruption of hydrogen bonding in cell membrane may be used as therapeutic index for drug monitoring.

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