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Thermodynamics of fluoxetine partitioning into lipid membranes

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Abstract: The partition coefficients (K_p) of fluoxetine, a well prescribed SSRI antidepressant, between zwitterionic1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), negative charge 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) large unilamellar vesicles (LUVs, 100 nm) and water were determined over a temperature range of 25℃-37℃ using second derivative spectrophotometric method. Fluoxetine was found to partition to a greater extent into the two DPPC and DPPG LUVs with increasing temperature. In addition, the partition coefficient of fluoxetine into DPPG LUVs was higher than into DPPC LUVs, indicating the positively charged drug molecules are highly accessible to the negatively charged DPPG bilayers. Van't Hoff analysis of the temperature dependence of K_p value revealed positive values for both ΔH and ΔS, suggesting an entropy driven mechanism for fluoxetine partitioning into DPPC and DPPG LUVs. An in-depth study of the thermodynamic characterization of fluoxetine partitioning can shed light onto understanding the energetic forces driving the drug molecules to interact with lipid membranes.

Keywords: *Fluoxetine, Large Unilamellar Vesicles, Thermodynamics, Partitioning Coefficient, Second Derivative Spectrophotometry*

Introduction:

Fluoxetine is one of the most widely prescribed antidepressants because of its less toxicity and side-effects compared to other antidepressants [1]. It belongs to SSIR group that delays the reuptake of serotonin neurotransmitter by blocking the action of the serotonin transporter [2, 3]. Recently, it was found that the action of fluoxetine also correlates with its interaction with the ion channel TREK-1, which is embedded in cell membranes [4, 5]. The inhibition of fluoxetine on the ion channel protein TREK-1 is dissociating C-terminal domain of the protein, which causes the channel to be lost its function, results in the depression-resistant phenotype [4, 5]. In order to do that, fluoxetine must partition into the interior of the lipid bilayers, which in turn affects the protein function and efficacy of the drug. Therefore, studying the partition mechanism of fluoxetine into lipid membranes plays a vital role in drug design and development. Thermodynamics can reveal not only essential driving forces of drug partitioning into lipid bilayer membranes but also helpful information for their pharmacodynamic and pharmacokinetic understanding [6-8]. A key part of drug design and development is the optimization of molecular interactions between an engineered drug candidate and its binding target. Thermodynamic characterization provides information about the balance of energetic forces driving binding interactions and is essential for understanding and optimizing molecular interactions of drug and lipid membranes [8]. However, there is only few previous studies investigated on the interaction of fluoxetine with phosphatidylcholine liposomes [9] by DSC and no such information is available on the thermodynamic behavior of fluoxetine and its interaction with different kind of lipid membranes. Therefore, studying the thermodynamic characterization of fluoxetine partition can shed light onto understanding the energetic forces that drive the binding interactions and the essential for understanding and optimizing molecular interactions of fluoxetine into lipid membranes, an important key for drug design. In order to study the partitioning of fluoxetine, partition coefficient (K_p) is one of the powerful indicators for drug distribution into lipid membranes. In previous studies, the partition coefficients were usually determined using an isotropic two-phase solvent system such as an n-octanol/water mixture [10-12]. However, the n-octanol/water system is too simple to study drug and membrane interactions, thus the development of liposomes allows the studies pharmacological and toxicological behavior of drugs [13, 14]. Liposomes have dramatically become a widely used model for biological membranes to predict partition coefficients of drugs [15-18], since they mimic better the inner hydrophobic part and the outer polar, charged surface of the phospholipids of natural membranes. The main phospholipids found in biological membranes are glycerophospholipids, thus synthetic phospholipids such as zwitterionic 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), or ionic 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) were often used in many studies of drug and lipid bilayer interactions [19, 20]. Determination of the partition coefficients of drugs in liposome/buffer systems has usually been accompanied by separation procedures of centrifugation or filtration or membrane dialysis [21]. However, these separation procedures are troublesome and may disturb the equilibrium states of the sample solutions and also lead to errors arising from non-specific matrix adsorption of drugs onto membranes. It has been recognized that derivative spectrophotometry is applicable to the determination of partition coefficients without any separation procedures since it can eliminate the effect of

background signals and hence does not require optically clear sample solutions [22, 23]. By using the liposome/water system, and applying second derivative spectrophotometry, the partition coefficients of widely used psychotropic phenothiazine drugs, chlorpromazine and promazine were determined [24]. Fluoxetine with pK_a of 10.1 [25] will carry a net positive charge at physiological pH. Therefore, the electrostatic interactions between fluoxetine and the negatively charged DPPG LUVs can play an important role in governing the partitioning of the drug into the lipid vesicles as well. Besides, thermodynamics was studied as one of the essential driving forces of drug partitioning into lipid bilayer membranes [26, 27]. It provides fully understanding for drug pharmacodynamics and pharmacokinetics. In this study, the thermodynamics of fluoxetine partitioning into 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol (DPPG) large unilamellar vesicles (LUVs) were examined using second derivative spectrophotometry.

Materials and Methods:

1. Materials:

Fluoxetine was purchased from Sigma Aldrich (USA) and used as recieved. 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine C16:0 (DPPC), 1,2-dipalmitoyl-*sn*-glycero-3-phosphoglycerol C16:0 (DPPG) was supplied by Avanti Polar-Lipids Inc. (USA). Nanopure water distilled from Nanopure system with impedance of 18 MΩ, was used to prepare all solutes during the experiments. All liquid suspensions were made with Hepes buffer (NaCl 50 mM, pH 7.4).

2. Methods:

2.1. Liposome Preparation:

Chloroform in lipid solution was evaporated under nitrogen stream. Further removal of the solvent residue was performed by applying a high vacuum at room temperature for more than 4 hours. Then
the dried lipid film were stored at -20°C for further using. The resulting dried lipid film was dispersed in HEPES buffer (50 mM NaCl, pH 7.4) and the mixture was vortexed above the phase transition temperature to produce homogeneous multilamellar

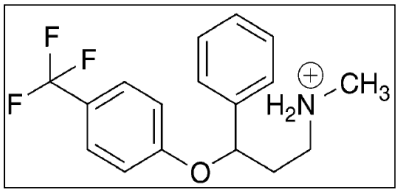


Figure 1. Chemical structure of fluoxetine

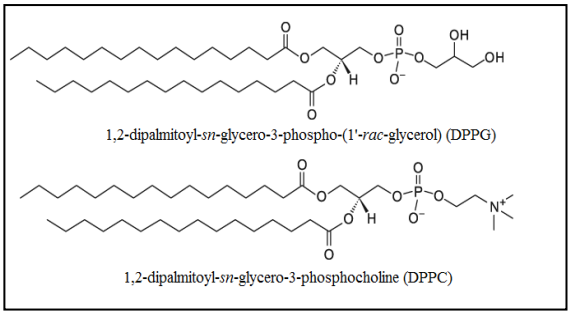


Figure 2. Chemical structures of DPPG and DPPC

(MLV). Frozen and thawed MLVs were obtained by repeating five times a cycle of freezing the vesicles in -20℃ cold store and then thawing in a water bath at 80°C. Large unilamellar vesicles (LUVs) with a diameter of 100 nm were prepared by extrusion the lipid suspension 30 cycles by using the polycarbonate filters with 100-nm pore size.

2.2. Absorption and Second Derivative Spectrophotometry

Two sets of several 1ml eppendorf were provided (one for the sample solutions and the other for the reference solutions). The buffer was added to each sample and reference eppendorfs and then a suitable aliquot vesicle suspension was further added to 800µL volume of solutions. To the eppendorf for sample solutions an approriated amount of drug was added so that the final drug concentration was 67.5 µM. Each eppendorf was vortexed for a short time and incubated at 37^{o}C (following the 32^{o}C and 25^{o}C experiments was tested) for 45 minutes. An absorption spectrum was measured against the reference solution by using a microcell cuvette with the chamber volume of 700 µL on the Agilent Cary 60 UV-Vis spectrophotometer with a temperature-regulated cell holder. The spectral window was set up from 190 nm to 300 nm. The second derivative spectra were calculated using a Origin program based on the Savitzky-Golay method [28], in which the second-order polynomial convolution of 20 points was employed. A wavelength interval ($\Delta\lambda$) of 1 nm was employed in the calculation.

2.3. Calculation of Partition Coefficients

The main disadvantage of using that spectroscopic method in investigation of drug-lipid interactions in liposome suspensions is high background signals derived from light scattering overlapping drug signals are eliminated. However the counterbalance of background signal by measurements of absorption spectra of investigated samples against the reference with the same lipid concentration is not complete. It might be done only by use the second derivatives of absorption spectra.

The molar partition coefficient of drug between lipid bilayer vesicle suspensions and aqueous solution is defined as

$$K_p = \frac{fraction\ of\ drug\ in\ lipid/[L]}{f\ raction\ of\ drug\ in\ water/[W]} \quad (1)$$

[L]: lipid molar concentration
[W]: water molar concentration
Fraction of the bound drug is defined as $\Delta D/\Delta D_{max}$, where

$$\Delta D = D - D_0 \quad (2)$$

is proportional to the fluoxetine concentration in the membrane [24]. *D* represents the intensity of the second derivative of absorption spectrum of fluoxetine solution in the presence of LUVs, whereas D_0 is the same intensity for pure fluoxetine solution. At enough high lipid concentrations, i.e. when 100% of fluoxetine is bound to the lipid, ΔD reaches its maximal value ΔD_{max}.
From equation (1) one can obtain

$$K_p = \frac{(\Delta D)/(\Delta D_{max})[W]}{(1-(\Delta D)/(\Delta D_{max}))[L]} \quad (3)$$

After simple transformations of the equation (3), equation (4) is obtained following:

$$\Delta D = \frac{K_P \Delta D_{max}\,[L]}{[W] + K_P[L]} \quad (4)$$

The values of the partition coefficients K_p, can be calculated from the experimental values of ΔD and [L] for a given drug concentration, by applying a non-linear least-squares calculation to equation 4. In this study, the second derivative spectra were calculated by Origin 8.5.1 software (OriginLab, Northampton, MA) and the Kp value were calculated by SigmaPlot 11.0 software.

2.4. Calculations of Thermodynamic Parameters

Thermodynamics of the partitioning of drug into the lipid bilayers are determined from the equilibrium constant K_p by the relationship between changes in Gibbs free energy (*ΔG*) and the logarithm of equilibrium constant LnK_p:

$$\Delta G = -RTLnK_P \quad (5)$$

Where R is the gas constant and T is temperature.

The change in free energy *ΔG* corresponding to the change in enthalpy *ΔH* minus the change in entropy *ΔS* at a certain temperature T:

$$\Delta G = \Delta H - T\Delta S \quad (6)$$

Van't Hoff equation is established by substituting equation (6) into (5):

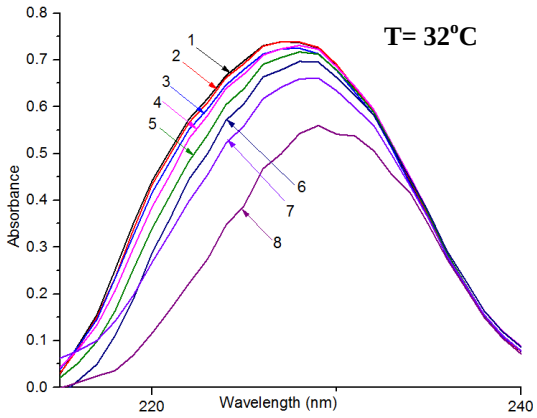
$$LnK_p = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (7)$$

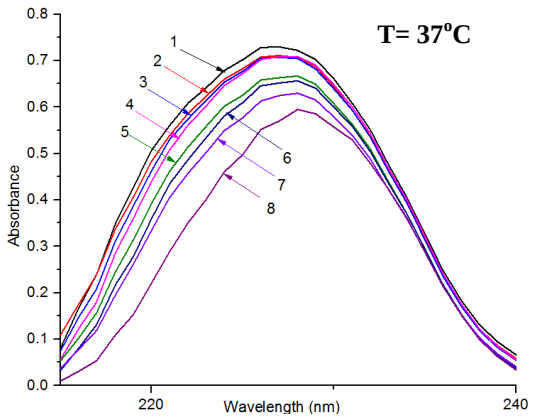
A straight line is set by the LnK_p plotted against 1/T, and then the enthalpy change *ΔH* and entropy change *ΔS* values will be determined from the slope of the straight line (-*ΔH*/R) and roll angle (*ΔS*/R), respectively.

Results and Discussion:

1. Absorption spectra of fluoxetine in DPPC & DPPG:

The absorption spectra of fluoxetine at a concentration of 67.5 μM in the presence of various amounts of lipid vesicles containing DPPC, DPPG at three temperatures 37°C, 32°C and 25°C were depicted in Figure 3 and Figure 4. It is important to point out that the concentration of fluoxetine used in this study was obeyed Beer's Law for absorption spectra.
The curves (2-8) in Figure 3 and Figure 4 were obtained by subtracting the absorption spectrum of lipid without fluoxetine (blank solution) from the absorption spectrum of lipid with fluoxetine recorded at the same lipid concentration. When increasing the lipid concentration of DPPC and DPPG the absorption maxima shifted to longer wavelength (bathochromic shift) as compared to the maximum in the buffer solution (spectrum 1). The bathochromic shift of fluoxetine in the presence of DPPC lipid was caused by decreasing of polarity in the surroundings of fluoxetine molecules, revealing the partitioning of fluoxetine into the lipid bilayer [16, 29]. The bathochromic shift was also observed previously for phenothiazine [29], chlorpromazine and methochlorpromazine [30] and trifluoperazine [31] in low polar solvents and for trifluopromazine [32] and promazine [24] in the presence of PC liposomes.





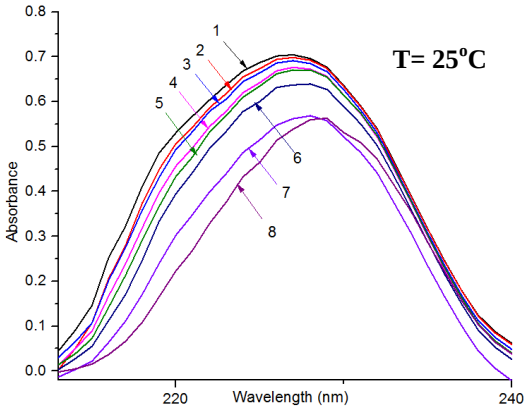


Figure 3. Absorption spectra of 67.5 μM fluoxetine in Hepes (pH 7.4) containing various amounts of DPPC LUV at 37°C, 32°C and 25°C respectively. Lipid concentrations mM: (1) 0, (2) 0.0169, (3) 0.0338, (4) 0.0675, (5) 0.1013, (6) 0.1350, (7) 0.2015, (8) 0.3375

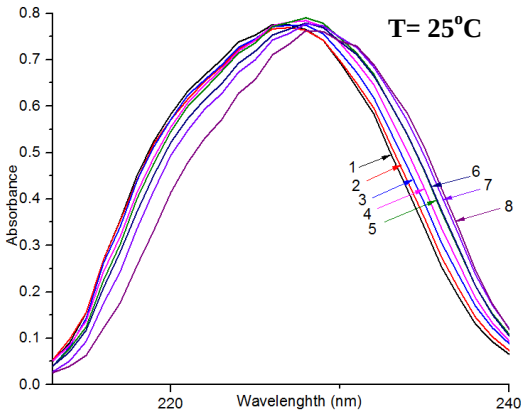
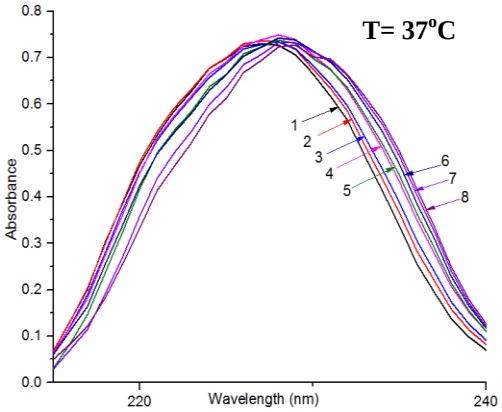
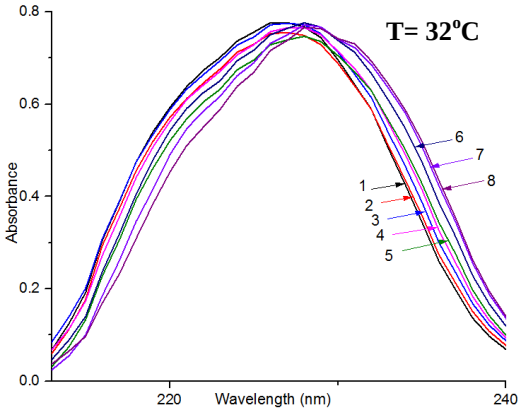


Figure 4. Absorption spectra of 67.5 μM fluoxetine in Hepes (pH 7.4) containing various amounts of DPPGLUV at 37°C, 32°C and 25°C respectively. Lipid concentrations mM: (1) 0, (2) 0.0169, (3) 0.0338, (4) 0.0675, (5) 0.1013, (6) 0.1350, (7) 0.2015, (8) 0.3375.





The absorption spectra of fluoxetine in the sample solution containing various mounts of DPPC and DPPG LUVs at three temperatures were shown in Figure 3 and Figure 4. The absorption maximum of fluoxetine exhibits a bathochromic shift according to the increase in lipid concentration indicating the partition of fluoxetine to the lipid bilayer of LUV. However, obtaining the result without the counterbalance of the background signals of LUV in the sample and reference solutions, isosbestic points must be observed. Despite the fact that the solutions in the sample and reference cuvettes were prepared to contain the same amount of LUV. But any isosbestic point cannot be observed in these figures. It is obvious that strong background signals impede the complete base line correction. Thus, spectra data to calculate the partition coefficient values could not be obtained from these absorption spectra. To eliminate the background effects, scientists used second derivative spectra of absorption to observe isosbestic points. For example the study on thermodynamics of partitioning of phenothiazine drugs between phosphatidylcholine bilayer vesicles and water studied by second-derivative spectrophotometry [26] and determination of drug partition coefficients in dimyristoyl-L-phosphatidylglycerol DMPG/ liposomes [16].

1. Second derivative spectra of absorption

The second derivative spectra calculated from the absorption spectra of fluoxetine in saturated negative charge lipid DPPG at 37°C in Figure 4 were depicted in Figure 5. In contrast to Figure 4, Figure 5 exhibits a bathochromic shift with two derivative isosbestic points clearly observed that proving the residual background signal effects can be entirely eliminated.

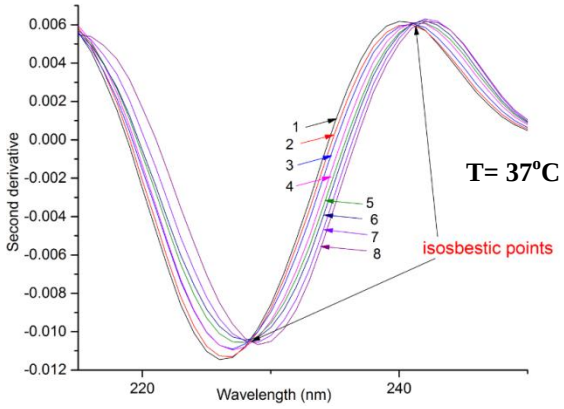


Figure 5. Second Derivative Absorbance Spectra calculated from Figure 4. Lipid concentrations mM: (1) 0, (2) 0.0169, (3) 0.0338, (4) 0.0675, (5) 0.1013, (6) 0.1350, (7) 0.2015, (8) 0.3375

2. Temperature Dependence of Partitioning

The ΔD values for fluoxetine in DPPC and DPPG LUVs were obtained from the derivative values at the wavelength of 220 nm. Using the ΔD values at different lipid concentrations the K_p values of fluoxetine at each temperature were calculated. The calculated K_p values are summarized in Table 1.

Table 1. The temperature dependence of K_p of fluoxetine between LUVs and buffer (pH 7.4)

Temperature (°C)	K_p (x10^5)	
	DPPC	DPPG
25	0.49±0.06	0.68±0.14
32	0.59±0.11	0.94±0.17
37	0.75±0.08	1.94±0.20

Each value is expressed as the mean ±S.D (n=3)

The calculated lipid/buffer partition coefficients (K_p) of all studied fluoxetine were within the range of 10^5 of magnitude. It was found that the partition coefficients of fluoxetine bind into DPPG is higher than the partition coefficients of fluoxetine into DPPC at all temperatures studied. These two lipids (DPPC and DPPG) are saturated and possess the same carbon number in their tails. At the temperature range from 25°C to 37°C they are both in the solid-gel state. They are just different in the head group in which DPPC is a zwitterionic lipid while DPPG is a negative charged lipid. Therefore, the higher value of K_p for partitioning of fluoxetine into DPPG than DPPC can be attributed to the electrostatic interaction between the positive charge of fluoxetine and negative charge of DPPG LUVs. This behavior is consistent with other a previous study about the interactions of ciprofloxacin drug (amphiphilic drug) with DPPC and DPPG large unilamellar vesicles. The partition coefficient value of ciprofloxacin into DPPC was less 3.5 times than DPPG [33]. In study here, the partition coefficient result in Table 1 showed that the partitioning of fluoxetine into the DPPG is higher than DPPC vesicles about 2.6 times at the temperature 37°C, 1.6 times at 32°C and 1.4 times at 25°C.

When the temperature was increased from 25°C to 37°C, the K_p values of fluoxetine into DPPC and DPPG LUVs were increased. The increase in K_p can be explained that at the higher temperatures, the lipids become more flexible and mobile so the fluoxetine molecules can partition more easily into the lipids. Lipids can exist in several lamellar phases depending on the temperature. For saturated phosphatidylcholines, such as DPPC, there are four recognized lamellar phases namely a liquid-crystalline phase (Lα), and phases with ordered hydrocarbon chain arrangements: ripple phase(Pβ); gel phase (Lβ); and subgel or crystal phase (Lc) [34]. The phase transition in lipid bilayers involves a cooperative structural change from a state in which the lipids are closely packed and their chains fully extended, to a state in which a large fraction of the molecules exhibits as many gauche rotations per molecule [33]. Therefore, when the temperature increases lipids will change their phase from the closely packed phase to more flexible phase (more disorder in the tail regions). The more flexible lipids have more space that make fluoxetine can bind to the lipids more easily than the lipids in the packed phase.

4. Thermodynamic Parameters

The thermodynamic parameters for fluoxetine partitioning from the water to the DPPC and DPPG lipid bilayers were calculated from the van't Hoff analysis based on the temperature dependence of K_p value. The van't Hoff plot for fluoxetine partitioning into DPPC and DPPG were shown in Figure 6 and Figure 7, respectively.

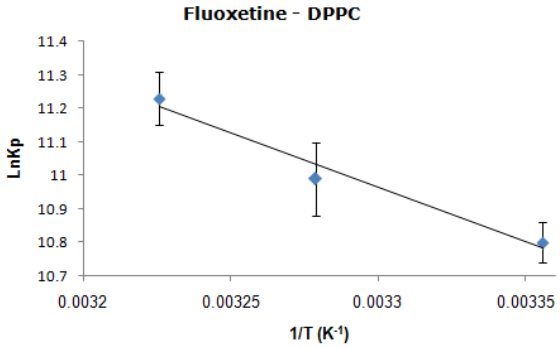


Figure 6. Van't Hoff plot for fluoxetine partitioning into DPPC LUVs.

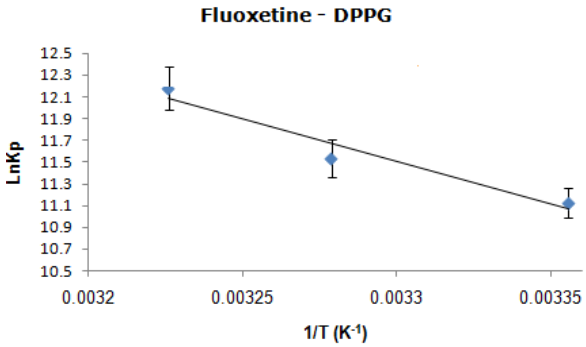


Figure 7. Van't Hoff plot for fluoxetine partitioning into DPPG LUVs.

Table 2. The thermodynamics of fluoxetine partitioning into DPPC and DPPG LUVs

Lipid	**ΔH (x10^4) (kJ/mol)**	**ΔS (J/mol K)**	**ΔG(x10^4) * (kJ/mol)**
DPPC	2.7±0.01	180.3±0.3	-2.7±0.03
DPPG	6.6±0.03	312.1±1.0	-2.8±0.03

∗ΔG values calculated at 25^{o}C.

The thermodynamic parameters obtained for fluoxetine partioning into DPPC and DPPG LUVs were listed in Table 2. The negative values of ΔG indicate that the partitioning of fluoxetine from water phase into DPPC and DPPG LUVs is thermodynamically favourable. The enthalpy change ΔH and entropy change ΔS were found to be positive when fluoxetine partitions into both DPPC and DPPG LUVs. It means entropy dominantly drives the partitioning of fluoxetine into the lipid bilayers. The entropy change obtained for fluoxetine partitioning into DPPG (ΔS =312.1 J/mol K) is nearly as twice as the entropy change for DPPC (ΔS =180.3 J/mol K), revealing that the electrostatic interaction between the positive charged residues of fluoxetine and the negative charged headgroup of DPPG contribute more significantly to the more disorder state of the lipid when fluoxetine partitions into. The entropy driven process is associated with the hydrophobic interaction, in which the hydrophobic parts of fluoxetine interact with the hydrophobic tails of lipids [29]. The lipid disorder was caused by the formation of gauche conformers in the tail region of lipids with drug partitioning [33]. The previous study on the interactions of ciprofloxacin (amphiphilic drug) with DPPC and DPPG using ATR-FTIR showed ciprofloxacin increased the order of the acyl chains of DPPC but decreasedthe acyl chain order of DPPG below the transition phase temperature [33]. Moreover, it was found that agomelatine, an antidepressant, strongly interacts with zwitterionic DPPC and charged DPPG membranes [35] and the interaction between agomelatine and DPPC liposomes caused DPPC to go to an ordered state (less trans/gauche isomerization of fatty acyl). In DPPG liposomes however, its interaction with agomelatine caused the disordering of liposomes, meaning that the number of gauche conformers increased (acyl chain flexibility) in the chains [36].
In past work, the binding of amphiphilic peptides to the membrane mimics was shown to be enthalpy-driven in a small vesicle with a diameter of 30 nm, but entropy-driven in a large vesicle with a diameter of 400 nm [37]. Seelig and co-workers explained this difference as due to the difference in the degrees of packing of lipids, which directly related to the curvature of the membrane surface and thus the internal bilayer pressure. As the size of a vesicle increases and thus the curvature of the membrane surface decreases, the packing constraints of the lipids are relaxed and the internal tension increases [37]. Hence, considerably more energy is required to insert a foreign molecule between the lipids of a planar membrane, compensating in part or totally the gain in van der Waals energy [37]. Because of this reason, entropy contribution to the Gibbs free energy in a large size of vesicles should be large to compensate the enthalpy increment but in a small size of vesicles enthalpy contribution is dominant. They, however, concluded that even though there was an enthalpy-entropy compensation mechanism the molecular origin of this effect was not clear [37]. Generally, the entropy change of the amphiphilic peptide binding onto a LUV with a small surface curvature is positive due to the disordering of bulk water and membrane lipids [37-39], which leads to the classical hydrophobic mechanism.

Conclusion:

The thermodynamics of the fluoxetine partitioning into LUVs composed of DPPC and DPPG were investigated. It was found that the partition coefficients K_p of fluoxetine into both DPPC and DPPG lipid systems were increased with increasing temperature from 25^{o}C to 37^{o}C. The partitioning of fluoxetine into DPPC and DPPG LUVs were found to be both entropy-driven. This means that the fluoxetine partitioning is governed mainly by the lipophilic immobilization of the solute in the phospholipidic bilayers (the disorder of the hydrophobic tails of lipid membranes). This work thus provides molecular insights into the interaction between an antidepressant fluoxetine, as a drug model and lipid membranes. Since there is no published study yet on this topic, the findings obtained here would be very valuable for future studies on the SSRIs-lipid membrane interactions.

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