Effect of Phosphatidylserine Content on the Partition Coefficients of Diazepam and Flurazepam between Phosphatidylcholine–Phosphatidylserine Bilayer of Small Unilamellar Vesicles and Water Studied by Second Derivative Spectrophotometry

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The affinity of the psychotropic benzodiazepine drugs diazepam (DZ) and flurazepam (FZ) to phosphatidylserine (PS) was examined since PS is abundantly contained in brain membranes. The effect of PS content on the partition coefficients (K_ps) of these drugs between phosphatidylcholine (PC)–PS bilayer membranes of small unilamellar vesicles (SUV) and water was measured using second derivative spectrophotometry. The second derivative spectra of DZ and FZ measured in the solutions containing various amounts of PC–PS SUV clearly showed derivative isosbestic points and a distinct derivative intensity change depending on the amount of the SUV added. The derivative intensity differences (ΔD) of the drugs before and after addition of the SUV suspension were measured at a specific wavelength. Using the ΔD values, the K_p values were calculated and obtained with relative standard deviation of below 10%. The K_p values of both drugs increased according to the PS content in the PS–PC bilayer membranes of the SUV proving that both have higher affinity to the PC–PS bilayer membranes than to PC membranes. The effect was much larger for FZ, *i.e.*, the K_p value of FZ at 30 mol% PS content increased to about five times the value for the PC SUV. This can be explained by the fact that at the experimental pH of 7.4, 80% of FZ molecules are in a cationic form (pK_a=8.1), so that these molecules are highly accessible to the negatively charged PS molecules. The results support the rapid and high distribution of DZ and FZ in the central nervous system after their administration.

Key words benzodiazepine; partition coefficient; derivative spectrophotometry; phosphatidylserine; liposome

Benzodiazepine drugs have been extensively administered as hypnotics, muscle relaxants, anxiolytics and anticonvulsants.[1,2] To evaluate the lipophilicities of benzodiazepine drugs is very important for their pharmacodynamical and pharmacological understanding since lipophilicity of a drug concerns its absorption, membrane transport, distribution and accumulation in the body.[3] Lipophilicity is commonly expressed by a partition coefficient and traditionally an octanol/water system partition coefficient has been used as a measure of lipophilicity.[3] However, it has been pointed out that the partition coefficients derived from liposome/water systems are superior to those obtained from oil/water systems in many quantitative structure–activity relationship (QSAR) applications.[4—7] Moreover, the partition coefficients of drugs between lipid bilayer vesicles (liposomes) and the aqueous phase are of great importance in the investigation of the behavior of drugs towards biomembranes.[8—12] Thus, the partition coefficients of several benzodiazepine drugs in natural[12—14] and model[8,9,15] membranes have been investigated.

Most of these model membranes, however, are prepared from phosphatidylcholine, and for psychotropic drugs the effect of phosphatidylserine (PS) on their interactions with phospholipid bilayer membranes should be determined, because brain membranes contain an abundance of PS as a component.

Determination of the partition coefficients of drugs in liposome/buffer systems has usually been accompanied by separation procedures of centrifugation,[4,5,7,16] filtration[17] or membrane dialysis[6,8,9] before measuring the concentration of free drugs or the amount of bound drugs. 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.[18,19]

In this paper we examined the effect of PS content on the partition coefficients of the benzodiazepin drugs, diazepam (DZ) and flurazepam (FZ), between PC–PS bilayer membranes of small unilamellar vesicles (SUV) by using second derivative spectrophotometry.

Experimental

Reagents Diazepam and flurazepam dihydrochloride were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and used without further purification. L-α Phosphatidylcholine (egg yolk) was supplied as a 2% (w/v) chloroform solution by Avanti Polar-Lipids Inc. (U.S.A.) and L-α-phosphatidyl-L-serine (bovine brain) was obtained from Sigma as a 10% (w/v) solution of chloroform containing 5% methanol. Both solutions were stored at −30 °C. Purity of the phospholipids was confirmed by thin layer chromatography (TLC). Other chemicals were of analytical reagent grade.

Liposome Preparation Appropriate amounts of the PC and PS stock solutions were mixed and evaporated by using a rotary evaporator. The content of PS (mol%) in the PC–PS mixture was calculated as PS/(PC+PS)×100 using the volume of each stock solution withdrawn and each concentration. Further removal of the solvent residue was performed by applying a high vacuum at room temperature for more than 4 h. Thereafter, 5 ml of buffer was added and vortex mixed to make a homogeneous multilayer large liposome suspension. The buffer used was 50 mM NaCl–10 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes buffer, pH 7.4). The suspension was subsequently sonicated into SUV by an ultrasonicator UD-200 (Tomy Seiko Co. Ltd., Tokyo, Japan) at a power level of 5.0 under a gentle stream of nitrogen in an ice-water bath. Thirteen consecutive cycles

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of 3 min sonication with 3 min interval were repeated, making the net sonication time 39 min. The sonicated suspension was centrifuged for 20 min at 3500 rpm (2000×*g*) to eliminate sediment from the sonication tip.[10)]

Determination of Lipid Concentration The exact lipid concentration (PC+PS) in the SUV suspensions was calculated by phosphorus determination.[20)]

Absorption and Second Derivative Spectrophotometry Two sets of several 5-ml volumetric flasks were provided (one for the sample solutions and the other for the reference solutions) and 4 ml of buffer solution was added to each of them. To the flasks for sample solutions, 50 μl of a stock solution of 3.0 mM DZ or FZ was added, so that the final drug concentration was 30 μM. Then, a suitable aliquot of the vesicle suspension was added to each sample and reference flasks and the buffer was further added to volume. Each flask was shaken for a short time and incubated at 37 °C for 30 min. An absorption spectrum was then measured against the reference solution using 10 mm light-path length cuvettes at 37 °C with a slit width of 2 nm and a wavelength interval of 0.1 nm on a spectrophotometer (Hitachi U-3210) equipped with a temperature-regulated cell holder. The spectral data was transferred from the spectrophotometer to a personal computer (NEC PC-9801 VX) through an RS-232C interface. The second derivative spectra were calculated by the personal computer using a BASIC program[21)] based on the Savitzky–Golay method.[22)] A wavelength interval ($\Delta\lambda$) of 0.8 nm was employed in the calculation.

Calculation of Partition Coefficients The molar partition coefficient[10,15)] (K_p) of benzodiazepine between lipid bilayer and water (buffer) is defined as

$$K_p = \frac{([B_L]/[B_T])/[L]}{([B_W]/[B_T])/[W]} \quad (1)$$

where $[B_L]$ and $[B_W]$ represent the concentrations of benzodiazepine in lipid (PC–PS) bilayer membranes and water, respectively, $[B_T]$ equals the total amount of benzodiazepine added ($[B_T]=[B_L]+[B_W]$), and [L] and [W] are molar concentrations of lipid and water (55.3 M, 37 °C), respectively.

As the derivative intensity is proportional to the solute concentration, the derivative intensity of benzodiazepine (denoted as D) at a specific wavelength is represented as follows,

$$D=E_L[B_L]+E_W[B_W]$$

where E_L and E_W are the molar derivative intensities for $[B_L]$ and $[B_W]$, respectively. When E is defined as $E=E_L-E_W$, D can be written as

$$D=E_W[B_T]+E[B_L] \quad (2)$$

A new variable ΔD is introduced to represent the difference between D and $E_W[B_T]$ as

$$\Delta D=D-E_W[B_T] \quad (3)$$

From Eq. 2,

$$\Delta D=E[B_L] \quad (4)$$

Thus, the ΔD value is proportional to the concentration of benzodiazepine in the lipid bilayer membranes. Finally, from Eqs. 1 and 4, we get

$$\Delta D = \frac{\Delta D_{max} K_p [L]}{[W] + K_p [L]} \quad (5)$$

where $\Delta D_{max}=E[B_T]$.

The values of K_p and ΔD_{max} were calculated from the experimental values of [L] and ΔD by applying a non-linear least squares method (accompanying a Taylor expansion) to Eq. 5. The calculation was performed by a BASIC program.[10)]

Results and Discussion

Absorption and Second Derivative Spectra The absorption spectra of DZ and FZ in the sample solutions containing various amounts of PC–PS (20 mol% PS) SUV are depicted in Figs. 1a and b, respectively. Neither DZ nor FZ exhibits significant spectral changes upon the addition of SUV. It is also obvious that the counterbalance of the background signals of SUV in the sample and reference beams is incomplete, despite the fact that the solutions in the sample and reference cuvettes were prepared to contain the same amount of SUV. Such strong background signals impede the complete base line correction. Thus, spectral data to calculate the K_p values could not be obtained from these absorption spectra.

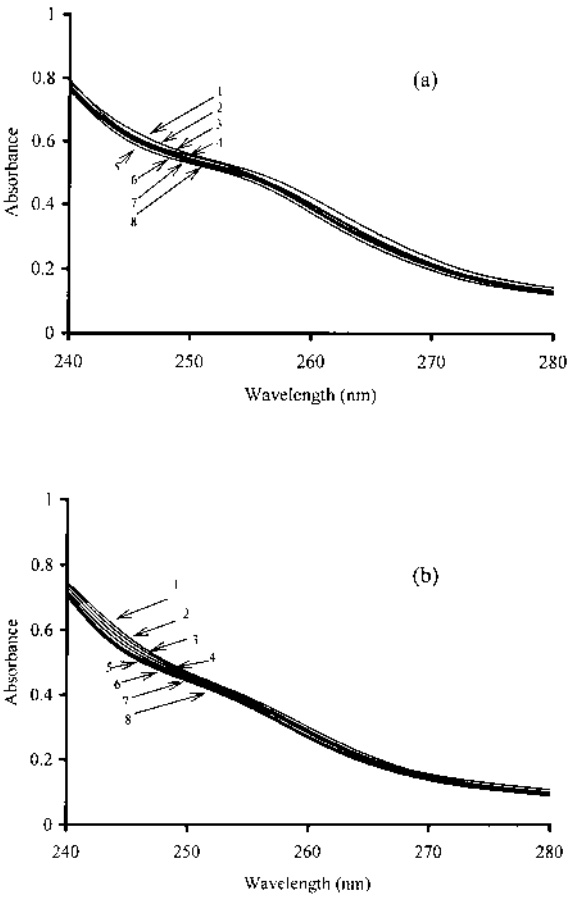

Fig. 1. Absorption Spectra of 30 μM Diazepam (a) and Flurazepam (b) in Hepes Buffer (pH 7.4, 37 °C) Containing Various Amounts of PC–PS (20 mol% PS) SUV

PC–PS concentration (mM): (a) 0 (1); 0.101 (2); 0.203 (3); 0.304 (4); 0.507 (5); 0.760 (6); 1.115 (7); 1.520 (8), (b) 0 (1); 0.15 (2); 0.30 (3); 0.45 (4); 0.75 (5); 1.13 (6); 1.65 (7); 2.25 (8).

The second derivative spectra calculated from the absorption spectra in Fig. 1 are depicted in Figs. 2a and b, respectively. In contrast to Fig. 1, both Figs. 2a and b exhibit a bathochromic shift and decrease in derivative intensity depending on the amount of SUV added. Also, three derivative isosbestic points are clearly observed for both drugs proving that the residual background signal effects can be entirely eliminated and that DZ or FZ exists in two states having different derivative spectra, *i.e.*, the drug exists in water and lipid bilayer phases.[23)]

Calculation of K_p and ΔD_{max} The ΔD values for DZ and FZ were obtained as the differences of the derivative values between the spectrum 1 and spectra 2—8 in Fig. 2a or b at the wavelength of 263 or 245 nm, respectively. At these wavelengths, large ΔD values could be obtained with good reproducibility.

To what extent the K_p values are affected by a small variation in the wavelength at which ΔD values are measured was confirmed by calculating the K_p values of DZ and FZ with ΔD values measured at 263±1 nm and 245±1 nm, respectively. Using these ΔD values and the lipid concentrations,

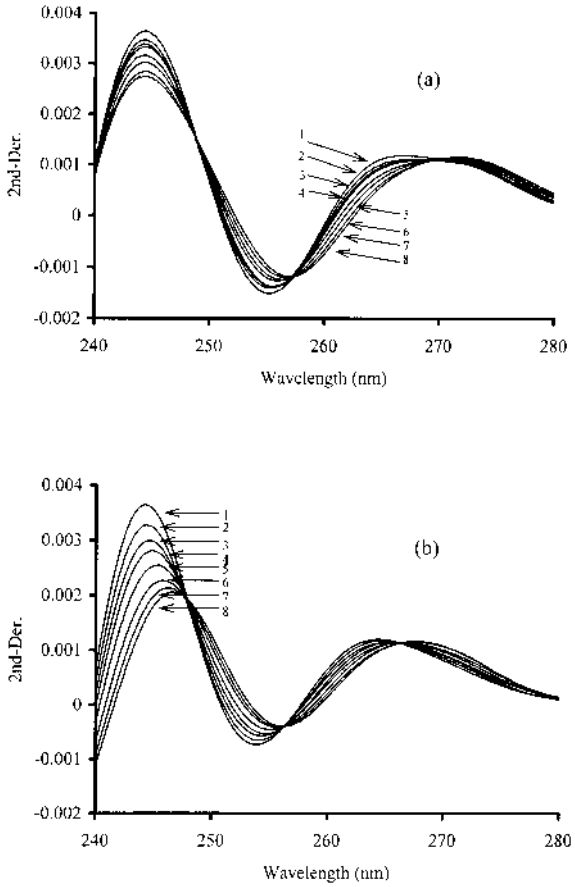

Fig. 2. Second Derivative Spectra of Diazepam (a) and Flurazepam (b) Calculated from the Absorption Spectra of Figs. 1a and b, Respectively

The numbers in the figures are the same as in Fig. 1.

the K_p and ΔD_{max} values were calculated by the non-linear least-squares method.

The results showed in Table 1 indicate that a small difference in these wavelengths does not affect the K_p values.

The effect of $\Delta\lambda$ value on the K_p value was also confirmed, since the $\Delta\lambda$ value affects the results of second derivative spectrum calculation.[21] The K_p values of both drugs were calculated by using the ΔD values measured from second derivative spectra obtained with four different $\Delta\lambda$ values (0.5, 0.6, 0.7, 0.8 nm). The results listed in Table 2 apparently show that the change in the $\Delta\lambda$ value has insignificant effect on the calculated K_p values. The $\Delta\lambda$ value of 0.8 nm has been employed in this study because of its lowest relative standard deviation (RSD).

These results confirm the robustness of the derivative method for the determination of partition coefficients of benzodiazepine drugs in a liposome/buffer system.

The K_p values at several PS contents were then calculated and are summarized in Table 3. For both DZ and FZ, the K_p values could be obtained with a RSD of below 10% indicating a good precision of the derivative method.

Effect of PS Content on the K_p Values The results in Table 3 show that the K_p values of both DZ and FZ apparently increase according to the increase in the content of PS in the bilayer membranes of PC–PS SUV. This proves that both drugs have higher affinity to PC–PS bilayer membranes than to PC membranes.

Table 1. Effect of Wavelength at which ΔD Values were measured on the K_p Values of DZ and FZ for the PC–PS (20 mol% PS) SUV

DZ		FZ	
Wavelength (nm)	K_p[a] ($\times 10^{-3}$)	Wavelength (nm)	K_p[a] ($\times 10^{-3}$)
262	35.1±1.9	244	46.7±2.0
263	36.9±1.1	245	50.5±2.0
264	38.5±1.6	246	50.0±2.2

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

Table 2. Effect of $\Delta\lambda$ on the K_p Values of DZ and FZ for the PC–PS (20 mol% PS) SUV Calculated from the ΔD Values at 263 and 245 nm, Respectively

$\Delta\lambda$ (nm)	$K_p\times(10^{-3})$[a]	
	DZ	FZ
0.5	35.5±2.4	51.4±3.3
0.6	37.0±2.5	53.0±2.7
0.7	35.7±2.2	51.1±2.7
0.8	36.9±1.1	50.5±2.0

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

Table 3. K_p Values of DZ and FZ for PC–PS SUV at Several PS Contents

PS (mol%)	$K_p\times(10^{-3})$[a]	
	DZ	FZ
0	30.8±2.5[b]	14.1±0.6[b]
10	32.6±1.8	30.5±0.6
20	36.9±1.1	50.5±2.0
30	42.5±2.4	68.2±1.7

a) Each value is expressed as the mean±S.D. (n=3). *b*) Refer to ref. 15.

A drastic increase in the K_p value of FZ was observed: though the K_p value of FZ for the PC SUV not containing PS is smaller than one half of the K_p value of DZ, FZ and DZ show similar affinity to the PC–PS membranes even at 10% content of PS. At 30% PS content FZ has an affinity about 4.8 times that for PC alone. This very pronounced preference of FZ to the PC–PS membranes can be explained as follows. Since pK_a of FZ is 8.1,[24] about 80% of the FZ added is in a protonated cationic form at the experimental pH 7.4, and the PS molecules in the PC–PS bilayer membrane of SUV are negatively charged, so that the cationic FZ can easily access the negatively charged surface of the PC–PS SUV.

However, though DZ is in a neutral form at pH 7.4 due to its pK_a value of 3.6,[25] the increase in the affinity of DZ to PS–PC bilayer membranes was also detected, thus further detailed investigations on the interactions of the benzodiazepine drugs with PS–PC bilayer membranes will be necessary.

The value of $\Delta D/\Delta D_{max}$, which corresponds to the fraction of the drug in PC–PS bilayer membranes, was calculated from Eq. 5 with the obtained K_p and ΔD_{max} values, and the results are shown as curves in Figs. 3a and b for 30 μM DZ and FZ, respectively. All curves show good fitness with the experimental data illustrating the precision of the derivative method.

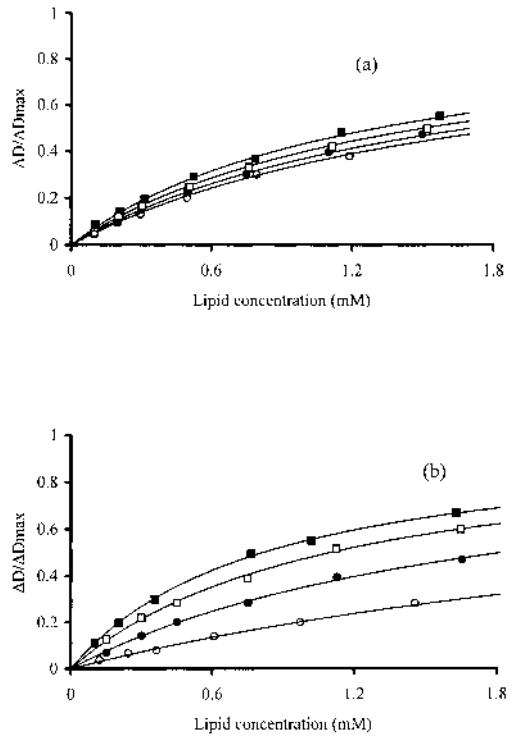

Fig. 3. Fraction ($\Delta D/\Delta D_{max}$) of Diazepam (a) and Flurazepam (b) in PC–PS SUV Membranes at Various PS Contents as a Function of Lipid (PC–PS) Concentration

The solid lines show the theoretical curves calculated from Eq. 5 using the experimental values of K_p and ΔD_{max}. The symbols are the experimental values. mol% of PS: (○) 0, (●) 10, (□) 20, (■) 30.

The results obtained in this study that DZ and FZ have a larger affinity to PC–PS membranes than to PC membranes support the fact of rapid and high distribution of the benzodiazepines in the central nervous system after their administration.

Though the benzodiazepine receptor is not lipid bilayer itself, it has been suggested that there are certain effects of benzodiazepines which may be generalized to all nerve cells and that can occur *in vitro* at micromolar concentrations.[12] These effects are nonspecific and mediated by interactions of benzodiazepines with lipophilic zones of biological membranes.[26] Thus the higher affinity of benzodiazepines to PS as compared to PC shown in this work supports the interactions with the lipid bilayer membranes of nerve cells.

In conclusion, to evaluate the affinity of benzodiazepines to biomembranes of the central nervous system by using a liposome/buffer system, it is highly preferable to use PC–PS liposomes rather than PC liposomes.

References

1) Söderpalm B., *Acta Psychiatr. Scand.*, **76**, 39—46 (1987).
2) Hardman J. G., Limbird L. E. (ed.), "Goodman & Gilman's The Pharmacological Basis of Therapeutics (9th ed.)," McGraw-Hill, New York, 1996, pp. 362—373.
3) Pliska V., Testa B., van de Waterbeemd H. (ed.), "Lipophilicity in Drug Action and Toxicology," VCH, Germany, 1996, p. 4.
4) Betageri G. V., Rogers J. A., *Int. J. Pharmaceut*., **46**, 95—102 (1988).
5) Choi Y. W., Rogers J. A., *Pharm. Res*., **7**, 508—512 (1990).
6) Rogers J. A., Choi Y. W., *Pharm. Res*., **10**, 913—917 (1993).
7) Pauletti G. M., Wunderli-Allenspach H., *Eur. J. Pharm. Sci*., **1**, 273—282 (1994).
8) Ottiger C., Wunderli-Allenspach H., *Eur. J. Pharm. Sci*., **5**, 223—231 (1997).
9) Ottiger C., Wunderli-Allenspach H., *Pharm. Res*., **16**, 643—650 (1999).
10) Kitamura K., Imayoshi N., Goto T., Shiro H., Mano T., Nakai Y., *Anal. Chim. Acta*, **304**, 101—106 (1995).
11) Kitamura K., Goto T., Kitade T., *Talanta*, **46**, 1433—1438 (1998).
12) Perillo M. A., Arc A., *J. Neurosci. Methods*, **36**, 203—208 (1991).
13) Perillo M. A., Garcia D. A., *Biomed. Chromatogr*., **6**, 183—190 (1992).
14) Perillo M. A., Garcia D. A., Arce A., *Mol. Membrane Biol*., **11**, 217—224 (1995).
15) Omran A. A., Kitamura K., Takagami S., El-Sayed A. Y., Abdel-Mottaleb, *J. Pharm. Biomed. Anal*., **25**, 319—324 (2001).
16) Zachowski A., Durand P., *Biochem. Biophys. Acta*, **937**, 411—416 (1988).
17) Luxnat M., Muller H.-J., Galla H.-J, *Biochem. J*., **224**, 1023—1026 (1984).
18) Talsky G., Mayring L., Kreuzer H., *Angew. Chem. Int. Ed. Engl*., **17**, 785—799 (1988).
19) Rojas F. S., Ojeda C. B., Pavon J. M. C., *Talanta*, **35**, 753—761 (1988).
20) Christopher A. J., Fennell T. R. F. W., *Microchem. J*., **12**, 593—605 (1967).
21) Kitamura K., Hozumi K., *Anal. Chim. Acta*, **172**, 111—118 (1985).
22) Savitzky A., Golay M. J. E., *Anal. Chem*., **36**, 1627—1639 (1964).
23) Connors K. A., "Binding Constants," John Wiley & Sons, New York, 1987, pp. 142—147.
24) Moffat A. C. (ed.), "Clarke's Isolation and Identification of Drugs in Pharmaceuticals, Body Fluids and Post-Mortem Material (2nd ed.)," The Pharmaceutical Press, London, 1986, p. 630.
25) Castro B., Gamerio P., *Anal. Chim. Acta*, **281**, 53—62 (1993).
26) Garcia D. A., Perillo M. A., *Biochim. Biophys. Acta*, **1418**, 221—231 (1999).