The Thermodynamics of Partitioning of Phenothiazines between Phosphate Buffer and the Lipid Phases of Cyclohexane, *n*-Octanol and DMPC Liposomes

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Abstract: The partitioning of six phenothiazines was determined between phosphate buffer (pH 6.0) and the lipid phases of cyclohexane, *n*-octanol and dimyristoyl phosphatidylcholine (DMPC). For DMPC liposomes studies were carried out both below and above the phase transition temperature (Tc) of the liposomes. The partitioning of chlorpromazine hydrochloride between *n*-octanol and phosphate buffer was both pH and concentration-dependent. A linear relationship between the absolute temperature (T^{-1}) and the logarithm of the equilibrium partition coefficient (ln K) was derived. The temperature dependence of the partition coefficient (K) over the temperature range 20–40 °C in cyclohexane and *n*-octanol, and 5–40 °C in DMPC liposomes, permitted the calculation of free-energy (G), enthalpy (H) and the entropy (S) of partitioning. Both the entropy and the enthalpy of partitioning of phenothiazines were positive in the three systems studied. In general, the partitioning of phenothiazines in cyclohexane, *n*-octanol and DMPC liposomes (both above and below the phase transition temperature (Tc)) is entropically controlled. Correlation was not however found between the free-energy of oil-water partitioning and liposome-water partitioning which may be attributed to the formation of surface associated phenothiazine in high concentrations at the liposome water interface. The concentration dependent partitioning of chlorpromazine in DMPC liposomes may be attributed to the adsorbed fraction of drug.

The pharmacological activity of a drug in the biological system in most cases requires that an interaction take place between the drug and one or more membranes. Liposomes have been widely studied as model biological membranes because these units consist of lipid bilayers, either multilayers arranged concentrically or a single bilayer encasing a volume of aqueous medium (1, 2). Thus, the study of interactions of drugs with aqueous dispersions of phospholipids can lead to a better understanding of the interactions of these drugs with biomembranes.

The work of Meyer (3) and Overton (4), who first showed the parallel relationship between the narcotic activity of drugs and their oil-water partition coefficient, initiated the use of partition coefficient measurements as a means for defining the relative hydrophobicity of biologically active organic compounds. Collander (5) generated new interest in oil-water partition coefficients by demonstrating that the rate of penetration of plant cell membranes by a wide variety of organic compounds was related to their partition coefficients. Octanol was considered as a possible reference solvent. Other studies (6–9) have since confirmed that polar hydrogen-bonding solvents are best suited to model hydrophobic substances reacting with biosystems. Hansch and Leo (10) initiated the fragment method of calculating partition coefficients, which as a thermodynamic property is a useful parameter for evaluating hydrophobic interactions of solutes with macromolecules, membranes, enzymes and drug receptors.

Murthy and Zografi (11) studied the partitioning of phenothiazines in dodecane and *n*-octanol, while Vezin and Florence (12) employed cyclohexane as the oil phase.

The distribution of a drug within a liposome depends on its structure and hydrophobicity. Drugs can be located in either the hydrophobic interior of the bilayer, in the aqueous channels or associated with the polar head groups at the surface of the bilayers. Using electron-spin resonance spectroscopy, perphenazine and chlorpromazine have been shown to be preferentially located in the polar part of the bilayer, whereas promethazine and oxidized derivatives of chlorpromazine are found principally in the hydrophobic interior of the bilayer (13). The ability of phenothiazines to alter the permeability of liposomes has been studied previously (14), and the interaction of chlorpromazine with liposomes determined (15, 16). An extensive study of the partitioning of phenothiazines into DPPC was reported (17). Ahmed et al. (18, 19) studied the partitioning and efflux of phenothiazines from liposomes and the interaction of mequitazine with liposomes (20).

In this paper, the partitioning of six phenothiazines was studied in three systems; cyclohexane and phosphate buffer (pH 6.0) *n*-octanol and phosphate buffer (pH 6.0) and DMPC liposomes and phosphate buffer (pH 6.0). The thermodynamics of partitioning were calculated and an attempt made to correlate the free-energy of oil-water partitioning to liposome-water partitioning.

Materials and Methods

Materials

The phenothiazines used were obtained as follows: Chlorpromazine hydrochloride (CPZ), Sigma Chemical Company Ltd., Poole, England); promethazine hydrochloride (PMZ), trimeprazine tartarate (TMZ) and prochlorperazine mesylate (PCZ), May and Baker Ltd., England); Trifluoperazine hydrochloride (TFZ), Smith, Kline and French Laboratories Ltd., U.K.) and fluphenazine hydrochloride

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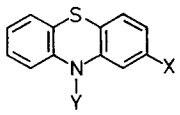
Table I. Chemical Structures of Phenothiazines.



Phenothiazine	X	Y	Structure	Molecular weight	$\lambda_{max.}$
Promethiazine HCl (PMZ)	H		$-CH_2-CH(CH_3)-N(CH_3)_2$	320.5	249
Trimeprazine Tartrate (TMZ)	H		$-CH_2-CH(CH_3)-CH_2-N(CH_3)_2$	746.0	252
Chlorpromazine HCl (CPZ)	Cl		$-CH_2-CH_2-CH_2-N(CH_3)_2$	355.3	254
Prochlorperazine Mesylate (PCZ)	Cl		$-CH_2-CH_2-CH_2-$piperazine$-CH_3$	566.1	258
Trifluoperazine HCl (TFZ)	CF_3		$-CH_2-CH_2-CH_2-$piperazine$-CH_3$	480.4	255
Fluphenazine HCl (FPZ)	CF_3		$-CH_2-CH_2-CH_2-$piperazine$-CH_2-CH_2-OH$	510.4	258

(FPZ), E. R. Squibb and Sons, England. All phenothiazines showed a single spot by thin layer chromatography (solvent system, 85 volumes of propan-2-ol and 15 volumes of water (21)).

Cyclohexane and *n*-octanol (BDH) were of analytical reagent quality and were used without further purification. Dimyristoyl phosphatidylcholine (DMPC), Sigma, showed a single spot by thin layer chromatography (21) (solvent system, 65 volumes of chloroform, 25 volumes of methanol and 4 volumes of water).

The chemical structure of the phenothiazines employed is given in Table I.

Methods

Oil-water partitioning

Equal volumes (10 ml) of the mutual saturated solvents, with the phenothiazines dissolved in the buffer solutions (5.63×10^{-5} mol l^{-1} unless otherwise stated), were placed together and shaken for 24 h using the shake-flask method. Care was taken to exclude light throughout the equilibration period. The two phases were then separated and the aqueous phase analysed by UV spectrophotometry (CE 292 Digital UV Spectrophotometer) at the maximum wave length of each drug (Table I). The amount of phenothiazine associated with the organic phase was found by difference and K calculated from a mean of three determinations. The partition coefficients were determined over the temperature range 20–40 °C.

Liposome-water partitioning

Phenothiazines (0.1 mg ml^{-1}) were prepared in 0.2 M phosphate buffer to produce aqueous solutions of pH 6.0. 5 ml of a 3 mg ml^{-1} stock solution of DMPC in chloroform was delivered to a 50 ml quickfit flask and the chloroform rapidly removed by rotary evaporation (Rotavapor R100, Buchi, Switzerland) at 40 °C. This resulted in a uniform dry lipid film being formed on the inside wall of the flask. Multilamellar liposomes were formed by transferring 10 ml of phenothiazine solution at 40° C to the flask containing the dried lipid film, along with 4 or 5 small glass beads, then swirling with the aid of a vortex mixer until all the lipid was dispersed. The flask was placed in a shaking water bath for 24 h, the minimum time required to reach equilibrium. Separation of the DMPC and aqueous phases was accomplished by centrifugation. The liposome preparation was transferred to a 10 ml centrifuge tube placed in a rotor equilibrated to the desired temperature. Centrifugation (MSE Prepspin 50 Ultracentrifuge, England) at 50,000 rev min^{-1} for 1 h resulted in phase separation. Samples of supernatant were carefully removed, diluted five times with phosphate buffer, pH. 6.0, and transferred directly to UV cuvettes. Absorbances were measured at the appropriate $\lambda_{max.}$ Thus, the partition coefficients were determined over the range 5–40 °C at intervals of 5 °C, with the liposomally associated phenothiazines calculated by difference. A concentration dependent study of chlorpromazine was undertaken over the sub-critical micelle concentration range of 5.6×10^{-6}M to 8.44×10^{-3}M.

Methodology

The partition coefficient of each phenothiazine between the oil or liposomal phase and the aqueous phase was calculated from the following expression:

$$K = \frac{C_o\ (mg\ ml^{-1})}{C_w\ (mg\ ml^{-1})} \quad (1)$$

where, C_o is the concentration of the phenothiazine in the oil phase, C_w is the concentration in the aqueous phase and F is the phase volume ratio. In the oil-water partitioning F = 1, while for the liposome-water partition, F = 666.67.

The temperature dependence of the equilibrium partition coefficient is given by the following relationship (22):

$$\text{Ln } K = \frac{\Delta S}{R} - \frac{\Delta H}{RT} \quad (2)$$

where, K is the equilibrium partition coefficient, H the enthalpy of partitioning, T the absolute temperature and R the gas constant (8.3143 J mol^{-1} K^{-1}). H can be found from the Van't Hoff plot of ln K versus T^{-1}, where the slope of the line is H/R and the intercept is the constant.

The free energy of partitioning is related to K by the equation:

$$\Delta G_{w\to l} = RT \text{ Ln } K \quad (3)$$

Once $\Delta H_{w\to l}$ and $\Delta G_{w\to l}$ are known, then $\Delta S_{w\to l}$, the entropy of partitioning can be calculated from the expression:

$$\Delta S_{w\to l} = \frac{\Delta H_{w\to l} - \Delta G_{w\to l}}{T}$$

Results and Discussion

The ln K for chlorpromazine hydrochloride (CPZ) partitioning between *n*-octanol and phosphate buffer increased as a function of increasing both CPZ concentration and the pH of the phosphate buffer from pH 5.8 to pH 7.8 (Fig. 1). The effect of pH on CPZ partitioning in *n*-octanol was studied to show the effect of the degree of ionization of the drug on partitioning. Table II shows that the partitioning of CPZ increased from pH 5.8 to pH 7.8. These results indicate that between pH 5.8 to pH 7.0 the ionic species of CPZ is partitioned as an ion-pair, while

Table II. The Effect of pH on Chlorpromazine Partitioning Between *n*-Octanol and Phosphate Buffer at 37° C. Mean ± S.D. (n=3)

pH	K	Ln K
5.8	338 ± 8.1	5.82 ± 0.14
6.0	470 ± 6.1	6.15 ± 0.08
6.2	607 ± 9.3	6.41 ± 0.10
6.6	892 ± 7.5	6.79 ± 0.06
7.0	1140 ± 10.9	7.04 ± 0.07
7.4	3090 ± 9.9	8.04 ± 0.03
7.8	4510 ± 10.7	8.41 ± 0.02

values above pH 7.0 represent in addition an increasing contribution of the free base. The linear increase in K with pH from 5.8 to 7.0 (r=0.999; n=5) may result from the increased concentration of HPO_4^{2-} compared to the $H_2PO_4^-$.

The reciprocal temperature dependence of ln K for the six phenothiazines is illustrated in Figures 2 and 3. The relationship of the thermodynamics of partitioning at 25 °C between the two oil-water systems, cyclohexane and phosphate buffer, pH 6.0, and *n*-octanol and phosphate buffer, pH 6.0, is given in Table III. The partitioning of phenothiazines in *n*-octanol was, with the exception of TMZ and PCZ, greater than in cyclohexane. The reason for this is due to a combination of the chemical structures of phenothiazines and that of the organic solvent studied. All the phenothiazines are water soluble, and the partitioning in the two systems depends on the drug hydrophobicity. In cyclohexane, K for TMZ is greater than PMZ because TMZ is more hydrophobic than PMZ. FPZ which is the least hydrophobic drug of this group, has, as expected, the smallest K value. This indicates that the only factor influencing the partitioning in cyclohexane is the hydrophobicity of the drug. Cyclohexane is the only solvent that is truly non-polar

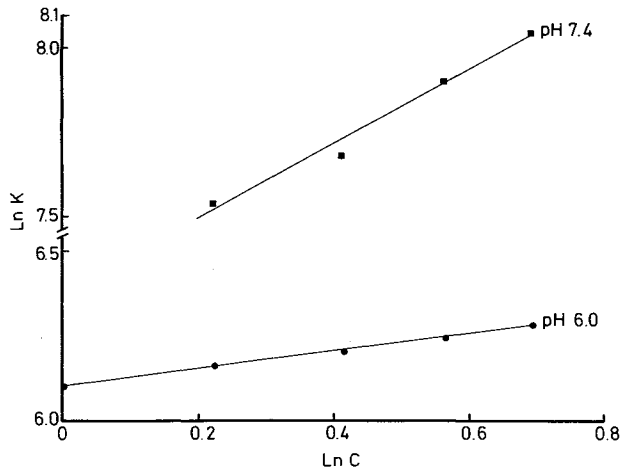


Fig. 1 The Ln C of CPZ as a Function of Ln K Between *n*-Octanol and Phosphate Buffer at 37°C.

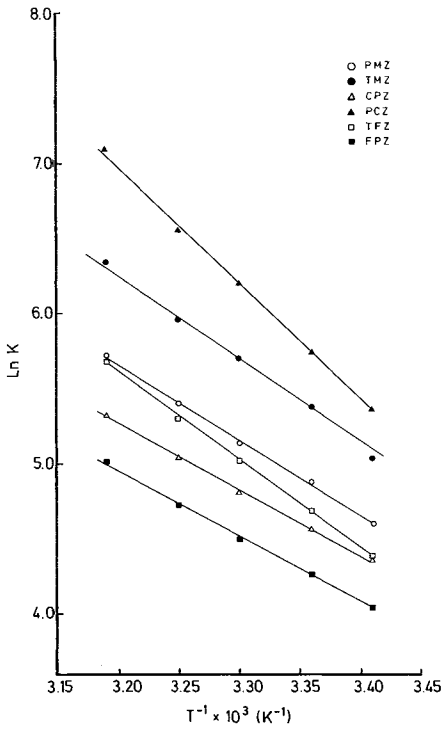


Fig. 2 The Partitioning of Phenothiazines Between Cyclohexane and Phosphate Buffer pH 6.0.

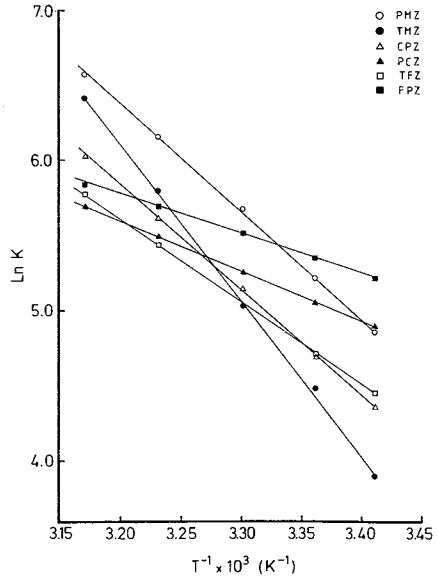


Fig. 3 The Partitioning of Phenothiazines Between *n*-Octanol and Phosphate Buffer pH 6.0.

Table III. Thermodynamics of Partitioning of Six Phenothiazines at 25° C. n=3; Max S.D. of mean ± 0.32 Ln K.

Phenothiazine	Cyclohexane-Phosphate Buffer pH 6.0				*n*-Octanol-Phosphate Buffer pH 6.0			
	Ln K	$\Delta G_{w\to1}$	$\Delta H_{w\to1}$	$\Delta S_{w\to1}$	Ln K	$\Delta G_{w\to1}$	$\Delta H_{w\to1}$	$\Delta S_{w\to1}$
PMZ	4.87	−12.1	41.9	181	5.22	−12.9	59.9	244
TMZ	5.37	−13.3	48.9	208	4.50	−11.2	86.6	328
CPZ	4.56	−11.3	35.9	158	4.70	−11.7	58.9	236
PCZ	5.75	−14.3	65.4	267	5.06	−12.5	28.3	137
TFZ	4.68	−11.6	49.4	204	4.72	−11.7	46.2	194
FPZ	4.25	−10.5	36.7	158	5.36	−13.3	22.0	118

Units: ΔG and ΔH→ kJ mol^{-1}; ΔS→ J mol^{-1} K^{-1}

and as such has no capacity for hydrogen bond formation. It therefore follows that cyclohexane has no tendency for self-association into molecular aggregates, there is no liquid structure, and it is an extremely poor solvent for water. Water exists only in a monomeric form in water-saturated cyclohexane. *n*-Octanol, in contrast, is a hydrophilic solvent and has a capacity for hydrogen-bonding and for self-association into aggregates (8). When water is added to *n*-octanol, there is a very strong tendency to form an aggregate of four alcohol molecules surrounding a central water molecule (A_4W). A water-saturated *n*-octanol binary phase may therefore have some advantages for drug partitioning in that a tightly structured lipoidal phase containing polar centres may be a better model of a biomembrane than the more thermodynamically acceptable hydrophobic cyclohexane. In water-saturated *n*-octanol, the partitioned phenothiazine molecule may replace one alcohol in an A_4W complex to maintain the equivalent of a monomeric dispersion of the partitioned phenothiazine.

Table III shows that both $\Delta S_{w\to1}$ and $\Delta H_{w\to1}$ are positive, although enthalpy-entropy compensation was not evident for these systems. The $\Delta G_{w\to1}$ values are large and negative for all phenothiazine drugs. It can be concluded that the partitioning of phenothiazines into cyclohexane and *n*-octanol over the temperature range of 20–40 °C is entropically dominated.

The partitioning of phenothiazines between DMPC liposomes and phosphate buffer, pH 6.0, over the temperature range of 5–40 °C at intervals of 5 °C is given in Fig. 4. K for all phenothiazines increases with temperature both below and above the Tc. There is a distinct break at or near 23 °C in the plot of ln K versus T^{-1} due to the change in phase of the DMPC liposomes from gel-crystalline to the liquid-crystalline state (23). The partition coefficients are also higher above Tc compared with values below the Tc (24). The partitioning of phenothiazines into DMPC liposomes is also found to be entropically controlled.

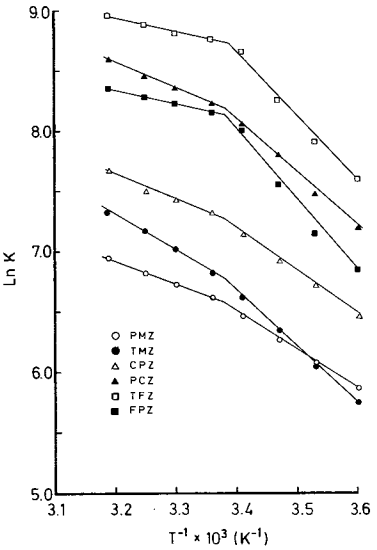


Fig. 4 The Partitioning of Phenothiazines Between DMPC Liposomes and Phosphate Buffer pH 6.0.

Table IV shows the calculated thermodynamic parameters of partitioning below Tc (15 °C) and above Tc (30 °C) in DMPC liposomes. For all phenothiazines, the free energy, enthalpy and entropy of partitioning are greater for the gel-crystalline phase than the liquid-crystalline phase of DMPC liposomes. The values of $\Delta G_{w\to1}$ for phenothiazines between DMPC liposomes and phosphate buffer, pH. 6.0, were considerably lower than that for both oil-water systems (Table V). These results may indicate that the ionized drug is adsorbed at the surface of the many bilayers which constitute the liposome. Although some phenothiazine molecules will inevitably partition into the bilayers, as evidenced by differential scanning

Table IV. The Thermodynamics of Partitioning of Phenothiazines Below Tc (15° C) and Above Tc (30° C) in DMPC Liposomes. n = 3; Max S.D. of mean ± 0.93 Ln K.

Phenothiazine	Below Tc (15° C)				Above Tc (30° C)			
	Ln K	$\Delta G_{w\to1}$	$\Delta H_{w\to1}$	$\Delta S_{w\to1}$	Ln K	$\Delta G_{w\to1}$	$\Delta H_{w\to1}$	$\Delta S_{w\to1}$
PMZ	6.27	−15.01	27.90	148.99	6.72	−16.93	16.89	111.62
TMZ	6.35	−15.21	39.84	191.15	7.02	−17.69	25.16	141.42
CPZ	6.95	−16.64	29.95	161.77	7.45	−18.77	17.33	119.14
PCZ	7.82	−18.73	38.35	198.19	8.37	−21.09	18.45	130.50
TFZ	8.24	−19.73	48.14	235.66	8.80	−22.17	11.48	111.06
FPZ	7.54	−18.05	52.22	243.99	8.22	−20.71	8.70	97.06

Units: ΔG and ΔH→ kJ mol^{-1}; ΔS → J mol^{-1} K^{-1}.

Table V. The Relationship Between the Free Energy of Partitioning of Phenothiazines in DMPC Liposomes, *n*-Octanol and Cyclohexane Both Below (20° C) and Above (35° C) Tc

Phenothiazine	ΔG of partitioning (kJ mol^{-1})					
	20 °C			35 °C		
	DMPC	*n*-Octanol	Cyclo-hexane	DMPC	*n*-Octanol	Cyclo-hexane
PMZ	−15.8	−11.2	−11.2	−17.5	−15.5	−13.8
TMZ	−16.1	−9.5	−12.3	−18.4	−14.4	−15.3
CPZ	−17.5	−10.6	−10.6	−19.3	−14.1	−12.9
PCZ	−19.6	−12.1	−13.1	−21.7	−13.9	−16.8
TFZ	−21.2	−10.8	−10.7	−22.7	−13.7	−13.6
FPZ	−19.5	−12.7	−9.8	−21.2	−14.5	−12.1

calorimetry (20), the major liposome-associated fraction is surface-associated. A concentration dependent study of CPZ distribution between DMPC liposomes and 0.2M phosphate buffer (pH 6.0) over the temperature range 5 to 40 °C was undertaken to define the CPZ sorption characteristics. Data for some of the temperatures studied are shown in Figure 5. Apparent partitioning increased with CPZ concentration to a maximum at 2.8×10^{-4}M. The decrease in K at higher CPZ concentrations may result from dissociation of the adsorbed CPZ complex at the outer bilayer surface. However, it is not clear why such dissociation should occur. A similar concentration dependence for CPZ has been previously reported for egg lecithin liposomes with a maximum at 1.4×10^{-4}M CPZ (18). Increasing the temperature resulted in a considerable increase in K at 2.8×10^{-4}M CPZ compared with the temperature dependence of K at, for example, 8.44×10^{-3}M. It is the bilayer-associated fraction that should be used in any calculation of partition coefficients for use in thermodynamic analysis, and hence, partition coefficients for amphipathic molecules such as the phenothiazines between liposomes and water as determined by a phase-separation technique have very limited value.

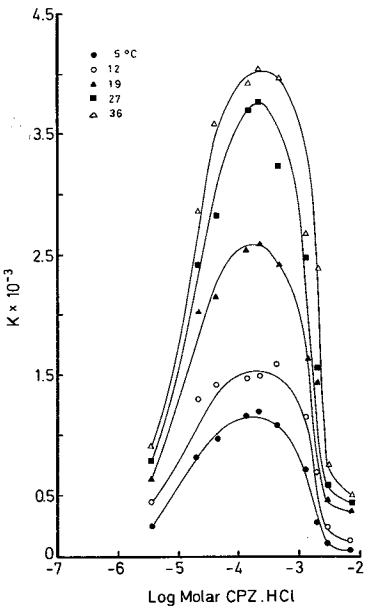


Fig. 5 The Relationship Between Equilibrium Partition Coefficient (K) and Varying Concentrations of CPZ.HCl in DMPC and 0.2M Phosphate Buffer (pH 6.0) at Different Temperatures.

Table VI. Enthalpies ($\Delta H_{w\to1}$), Free Energies ($\Delta G_{w\to1}$) and Entropies ($\Delta S_{w\to1}$) of Partitioning of CPZ Between DMPC/0.2M Phosphate Buffer (pH 6.0) Below and Above the Tc.

CPZ (M) Concentration	Below Tc			Above Tc			Tc (° C)
	$\Delta H_{w\to1}$ (kJ mol^{-1})	$\Delta G_{w\to1}$ (kJ mol^{-1})	$\Delta S_{w\to1}$ (J mol^{-1} K^{-1})	$\Delta H_{w\to1}$ (kJ mol^{-1})	$\Delta G_{w\to1}$ (kJ mol^{-1})	$\Delta S_{w\to1}$ (J mol^{-1} K^{-1})	
5.6×10^{-6}	42.49	−13.94	199.29	4.47	−17.04	70.38	22.4
2.8×10^{-5}	46.48	−16.66	222.92	14.10	−19.94	111.74	19.4
5.6×10^{-5}	39.58	−16.85	199.00	22.30	−20.47	139.90	19.4
1.4×10^{-4}	29.10	−17.10	163.18	22.00	−20.40	139.00	19.3
5.6×10^{-4}	36.55	−17.18	189.75	19.37	−20.74	131.57	19.0
4.22×10^{-3}	71.09	−12.42	294.93	13.47	−16.33	97.82	18.5
8.44×10^{-3}	110.08	−11.28	429.63	6.40	−15.61	71.63	17.7

The discontinuities in the plot of ln K as a function of T^{-1} may be taken as the Tc of DMPC liposomes in the presence of phenothiazine drugs, although the accuracy of such estimates arising from extrapolation procedures (Table VI) would be much less than direct determination by calorimetry. Both $\Delta H_{w\to1}$ and $\Delta S_{w\to1}$ are positive and $\Delta S_{w\to1}$ increases with increasing $\Delta H_{w\to1}$, whereas $\Delta G_{w\to1}$ is negative. It is also apparent that $\Delta H_{w\to1}$ and $\Delta S_{w\to1}$ are considerably higher below Tc for all concentrations of CPZ studied. CPZ therefore causes greater perturbation of membrane structure on transference from water to a liquid crystalline membrane than to the corresponding membrane in the gel state.

Correlation was not found between $\Delta G_{w\to1}$ for liposome partitioning and $\Delta G_{w\to1}$ for either of the lipoidal solvents (Table V), a fact which may be attributed to the formation of surface-associated phenothiazine at the liposome-water interface.

List of Abbreviations

Chlorpromazine hydrochloride	CPZ
Promethazine hydrochloride	PMZ
Trimeprazine tartrate	TMZ
Prochlorperazine mesylate	PCZ
Trifluoperazine hydrochloride	TFZ
Fluphenazine hydrochloride	FPZ
Dimyristoylphosphatidylcholine	DMPC
Equilibrium partition coefficient	K
Natural logarithm of equilibrium partition coefficient	LnK
Concentration of drug	C
Free-energy of partitioning	$\Delta G_{w\to1}$
Enthalpy of partitioning	$\Delta H_{w\to1}$
Entropy of partitioning	$\Delta S_{w\to1}$
Maximum wavelength for U.V.	$\lambda_{max.}$
Absolute temperature	T
Phase transition temperature	Tc

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Pharmacokinetic Study of Cefotaxime (CTX) in Dogs

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Abstract: Cefotaxime (CTX) was injected either intravenously or intramuscularly in dogs, and its pharmacokinetics in plasma and urine were determined with the use of HPLC assay. Cephalothin (CET) was administered in a similar manner as a reference agent. While both CTX and CET rapidly disappeared from plasma after intravenous injection, the half-life of CET was approximately 2.5 times shorter than that of CTX. Both drugs were deacetylated, and desacetyl-CTX and desacetyl-CET appeared in plasma. Both drugs were rapidly excreted into urine either in unchanged or deacetylated form, the sum of which accounted for 77 % and 63 % of the CTX and CET dose, respectively. The ratio of the amount of unchanged drug over that of deacetylated drug in the urine was 1:1 for CTX and 1:2 for CET. When CTX and CET were intramuscularly injected, the plasma levels of CTX and CET reached a maximum 30 min and 15 min after injection, respectively, followed by a rapid decline. The pattern of urinary CTX excretion was similar after i.m. and i.v. injections. In contrast, the amount of desacetyl-CET in the urine was larger after i.m. than i.v. injections. CTX metabolites other than desacetyl-CTX (M_2 and M_3) that were also assayed by HPLC accounted for only 2–4 % of the dose of CTX in the urine, but were below detectable levels in this plasma.

Cefotaxime (CTX, sodium 7-[2-(2-amino-4-thiazolyl)-2-methoxy-iminoacetamide] cephalosporanate) is a semi-synthetic cephalosporin with a broad antibacterial spectrum. A pharmacokinetic study of CTX in rabbits and rats has already been reported (1, 2). In this study, the pharmacokinetic profiles were determined for CTX and cephalothin (CET) as a reference drug with the same acetyl group as CTX. Furthermore, an attempt was made to determine CTX metabolites other than desacetyl-CTX. Dogs were used as an experimental animal in the present work.

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Materials and Methods

Chemicals

CTX Sodium salt, its desacetyl metabolite (desacetyl-CTX), and the desacetyl metabolite of CET (desacetyl-CET) were supplied by Hoechst AG. The CET sample used in this study was Keflin (Shionogi and Co., Ltd.).

Animals procedures

Male beagle dogs 9 to 20 months old, weighing 8.2 to 12.2 kg (CLEA Japan, Inc.), were used. Food was withdrawn 18 hours prior to the start of the experiments. Solutions of 20 % CTX and of 20 % CET were prepared with distilled water. Desacetyl-CTX was dissolved in 0.1 M phosphate buffer (pH 7.0) (2 % solution). The drug solutions were intravenously injected in one foreleg, and venous blood samples (1.5 ml) were collected from the other foreleg with a heparin-treated syringe. Samples were immediately centrifuged at 3,000 rpm for 10 minutes to separate the plasma.

Excreted urine was collected in a metabolic cage. After each collection, the cage was washed with a small amount of water. The urine remaining in the bladder was collected with a catheter. The excreted urine, the washings from the cage, and the urine collected with the catheter were combined as a urine sample at each time point.

HPLC analysis of CTX, desacetyl-CTX, CET, and desacetyl-CET

Samples were diluted with the HPLC eluent system, and 10 μl of the sample was injected into the column. Either the TRIROTAR system (Nippon Bunko Co., Ltd.) or fully automatic (Nihon Waters Limited) HPLC equipment was used. A column 4.6 mm in internal diameter and 12.5 cm in length, and filled with SC-02 (Jasco, 10 μ), was used. The following mobile