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Benzodiazepine localisation at the lipid-water interface: effect of membrane composition and drug chemical structure

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Abstract

The effect of membrane chemical composition and drug chemical structure on the localisation of several benzodiazepines (BZDs) (DZ, diazepam; CZ, clonazepam; CX, chlordiazepoxide) within model membranes was investigated. We used a spectrophotometric method presented in a previous paper (B.A. García, M.A. Perillo, Biochim. Biophys. Acta 1324 (1997) 76–84) based on the study of BZD acid-base equilibrium. 'Intrinsic p*K*' values (pK_i) were calculated according to the theory of M.S. Fernández and P. Fromherz (J. Phys. Chem. 81 (1977) 1755–1761). Homogeneous media of known dielectric constant (dioxane 0–80% v/v in water) were used to construct a curve of ΔpK_i ($pK_i - pK_w$) vs. dielectric constant (D) where ΔpK_i values obtained in lipidic dispersions were interpolated. In heterogeneous media consisting of aqueous dispersions of Triton X-100 micelles we determined the relative localisation depth of BZDs according to their D_{Triton} values (36, 37 and 62 for DZ, CX and CZ respectively) taking into account that lower D values correspond to deeper localisation. pK_i determined in dispersions of dipalmitoylphosphatidylcholine (dpPC) and egg phosphatidylcholine (egg-PC) mixed multilamellar vesicles showed that, when cholesterol content increased from 0 to 20 mole%, D values decreased (from 59 to 40) in dpPC vesicles and increased (from 51 to 72) in egg-PC vesicles, indicating a tendency of BZDs to penetrate deeper into less ordered interfaces. These results should be considered to understand the non-specific pharmacological effects of BZDs as well as to evaluate the actual relevance of their pharmacological concentrations.

Keywords: Benzodiazepine; Membrane phase state; Localization; Dielectric constant; Acid-base equilibrium; Spectrophotometry

1. Introduction

Benzodiazepines (BZDs) are a group of neuroactive drugs widely administered due to their hypnotic, muscle relaxant, anxiolytic and anticonvulsant effects [1]. It is widely accepted that the pharmacological actions of BZDs arise as a result of binding to the so-called 'specific sites' in membrane-bound proteins, whereas the lipid background is considered to play a more passive role [2–4]. However, being lipophilic drugs, they also interact non-specifically with biomembranes, reaching concentrations one order of magnitude higher than those in the surrounding medium [5,6] and affecting membrane structural properties [7–9]. Non-specific interactions may have important consequences for a wide variety of biological

Abbreviations: BZD, benzodiazepine; c.m.c., critical micellar concentration; CX, chlordiazepoxide; CZ, clonazepam; D, dielectric constant; dpPC, dipalmitoylphosphatidylcholine; DZ, diazepam; egg-PC, egg phosphatidylcholine; FNTZ, flunitrazepam; MLV, multilamellar vesicle; P, partition coefficient

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functions [10]; this type of interactions may mediate several effects of BZDs observed at global concentrations above their affinity for their specific receptors, such as some anticonvulsant actions [11,12] and their effects as inhibitors of calcium-calmodulin dependent protein kinase [13], voltage sensitive Ca^{2+} channels [14,15] and Na^{+} channels [15].

Non-specific interactions between membrane and lipophilic drugs can be described either by a Langmuir adsorption isotherm [16] or by a partition equilibrium model [6,17]. We have demonstrated that BZD-membrane interaction is consistent with the latter model since these drugs are incorporated in the membrane as an integral part of the bilayer [18,19]. Strong local accumulation of drugs within the plane of the membrane [10,20] may be a consequence of the lateral heterogeneity of membrane composition as well as the asymmetry between the two membrane leaflets [21,22]. Moreover, local drug concentrations may change in a dynamic way through its coupling to fluctuations of local molecular density.

It has been reported that a low-ordered molecular arrangement of the interface facilitates the partitioning of flunitrazepam (FNTZ) into membranes [19,23,24]; on the other hand, this drug is able to expand the phospholipid molecular area and to affect its surface geometry and electrostatics [24]. Changes in surface curvature and dipolar arrangements have been implicated in key phenomena related with cellular functions [25,26]. This supports the idea that in the phenomenon of partitioning, drug and membrane exert mutual effects, e.g. the drug may affect the physical state of the membrane [7–9, 27] and the initial physical state of the membrane may modulate the ability of the drug to reach the membrane and to penetrate into it [18,19,28].

In a previous work we developed a spectrophotometric method to determine the localisation of FNTZ, which is based on the study of its acid-base equilibrium in media of different polarity; our results analysed with the theory of Fernández and Fromherz [29] let us infer the final drug localisation within the lipid-water interface in membrane models (micelles or bilayers) [18]. In the present paper, by applying essentially the same method, we investigated the effect of membrane chemical composition and drug chemical structure on the localisation of several BZDs within the bilayer.

2. Materials and methods

2.1. Materials

Benzodiazepines (diazepam (DZ), chlordiazepoxide (CX) and clonazepam (CZ)) were from Hoffman La Roche (Basle, Switzerland). Phospholipids and cholesterol were from Sigma (St. Louis, MO, USA). Other drugs and solvents used were of analytical grade.

2.2. UV absorption spectra

The BZD solutions (15 μM final concentration) were prepared in aqueous dioxane solutions of different concentrations (0%, 20%, 45%, 70% and 82% of dioxane, v/v) in order to vary the polarity of the medium; pH values were adjusted between 0 and 10 for DZ, 0 and 12 for CX, 0 and 6 and 6 and 14 for CZ and measured with a Cole Parmer 59003 pH meter equipped with a glass electrode and a sensor for automatic temperature compensation.

Each BZD absorption spectrum was measured against a blank of the same solvent and identical pH used for dissolving BZD. Readings were recorded at 1 nm intervals within 200–400 nm wavelength range, using a Beckman DU 7500 double beam spectrophotometer equipped with a diode array detector and 0.0001 AU sensitivity.

The BZD spectrum was also determined in the presence of suspensions of either Triton X-100 micelles or liposomes of different phospholipids (L-α-dipalmitoylphosphatidylcholine (dpPC) or egg phosphatidylcholine (egg-PC)) with or without cholesterol in different proportions.

2.3. Lipid dispersions

Liposomes were prepared by evaporating, under reduced pressure, from a chlorophormic solution of dpPC or egg-PC (1.5 mg/ml final concentration) containing cholesterol (0–20 mole%). The dry lipid was suspended in water by repeating six consecutive cycles of heating for 2 min at 70°C (dpPC) or at 21°C (egg-PC), which are temperatures above the corresponding T_c of the lipid, and vortexing for 1 min. Triton X-100 (0.32 mM final concentration) was dispersed in water by the same method. In both

cases we used concentrations above the c.m.c. of the corresponding lipid; in these conditions, dpPC and egg-PC molecules self-aggregate into multilamellar vesicles (MLVs), and Triton X-100 into micelles [30].

2.4. Corrections of pH meter readings

Dioxane-water solutions with known stoichiometric proton concentrations were prepared by the addition of known amounts of HCl or NaOH from standardised solutions, and their pH values were calculated as $pH_{calc} = -\log[HCl]$ or $pH_{calc} = 14 + \log[NaOH]$ and measured as indicated above to obtain pH_{obs} values. Then, pH_{calc} values were plotted versus pH_{obs} values and the curves obtained were adjusted to second-degree equations on pH_{obs}, by non-linear regression using a computer-aided least squared method. The resulting function was used to correct pH_{obs} values in other experiments. This calibration allows elimination of the combined and indistinguishable contributions of 'primary medium effect' and the 'liquid junction potential' from the pH readings [31].

2.5. Calculation of BZD equilibrium dissociation constant

The method used for calculation of the equilibrium dissociation constant was described in a recent paper [18]. Here we show briefly some of the theoretical considerations.

BZDs undergo acid dissociation reactions like the following (see Fig. 1 for details):

$$BZDH^+ \rightleftarrows BZD + H^+ \quad (1)$$

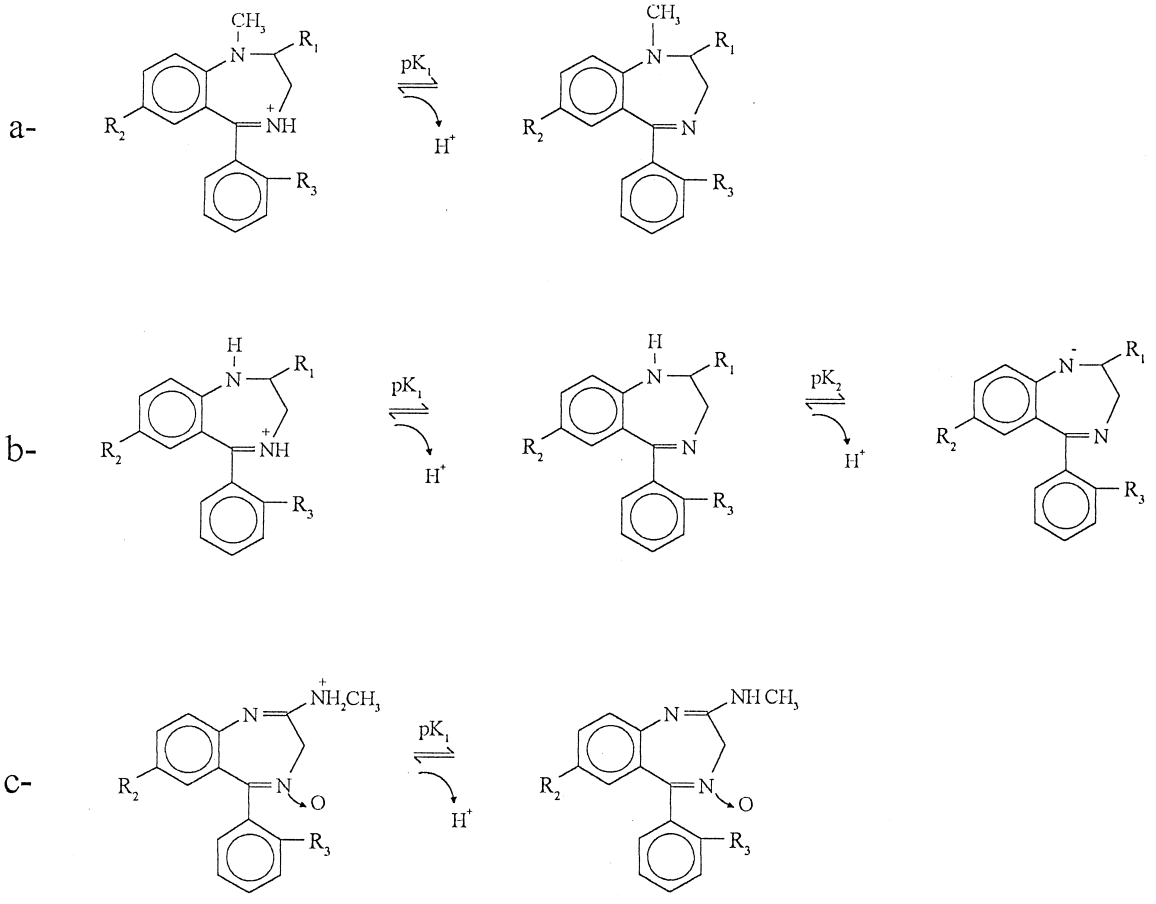

Fig. 1. Acid-base equilibrium reactions of benzodiazepines. 1,4-Benzodiazepin-2-ones: (a) DZ ($R_1 = O$, $R_2 = Cl$, $R_3 = H$) and FNTZ ($R_1 = O$, $R_2 = NO_2$, $R_3 = F$); (b) CZ ($R_1 = O$, $R_2 = NO_2$, $R_3 = Cl$). 4-*N*-Oxide-benzodiazepine: (c) CX ($R_2 = Cl$, $R_3 = H$).

The equilibrium dissociation constant (K) for this reaction has the form:

$$K = \frac{[BZD][H^+]}{[BZDH^+]} \quad (2)$$

In homogeneous systems such as dioxane-water mixtures, K can be calculated by measuring BZD absorbance as a function of pH and adjusting these experimental data to the following equation:

$$A_{obs}\frac{[H^+]\times A_{obs1} + K\times A_{obs2}}{[H^+] + K} \quad (3)$$

where A_{obs1} = absorbance of protonated form and A_{obs2} = absorbance of dissociated species.

In heterogeneous systems such as dispersions of lipids or detergents,

$$A_{obs} = A_m + A_w$$

where A_m and A_w are the absorbance values of the drug partitioned into the membrane and dissolved in water respectively. Then, Eq. 4 results in the sum of two expressions similar to Eq. 3:

$$A_{obs} = \frac{[H^+]\times A_{obs1m} + K_{app}\times A_{obs2m}}{[H^+] + K_{app}} + \frac{[H^+]\times A_{obs1w} + K_w\times A_{obs2w}}{[H^+] + K_w} \quad (4)$$

where $A_{obs1m} = A_{obs1} - A_{obs1w}$, $A_{obs2m} = A_{obs2} - A_{obs2w}$, K_{app} is the equilibrium dissociation constant in heterogeneous systems, and K_w is the equilibrium dissociation constant in water. A_{obs1} and A_{obs2} are experimental values of absorbance measured in the heterogeneous system at the extremes of the titration curve. A_{obs1w} and A_{obs2w} are absorbance values of the species BZD and $BZDH^+$ in water measured in a separate experiment at the extremes of the titration curve. In order to calculate K_{app}, the values of A_{obs} measured in lipid or detergent dispersions as a function of pH should be adjusted to Eq. 4.

The values of A_{obs1} and A_{obs2} can be corrected by drug partitioning into membrane using actual values of concentration in water which depend on individual partition coefficients (P_i) of protonated and non-protonated species. However, this has not been necessary under the present experimental conditions, since (a) BZDs have relatively low P values [32] and (b) due to the relatively low lipid concentration, the membrane volume was negligible compared with aqueous volume.

Then, from the values of K, pK values can be calculated as $pK = -\log K$.

2.6. Changes in pK values induced by changes in environmental polarity in self-aggregating structures of amphipathic molecules

Theory and methods used to analyse these results were essentially those developed by Fernández and Fromherz [29]. The titration of a drug in a medium of known polarity allows to determine 'intrinsic K' values (corresponding to pK_m in aqueous dioxane solutions or pK_w in water) which reflect the tendency of the drug to dissociate in the medium and can be calculated according to the actual concentrations, in those media, of all chemical species participating in the equilibrium (BZD, $BZDH^+$ and H^+).

pK_{app} values determined in a heterogeneous system as described previously (Eq. 4) correspond to 'interfacial' pK (pK_i) because the concentration of H^+ used for its calculation is the one in water and not the one in the membrane. If the interface is electrically charged, proton activity at the interface is different from that in the bulk of the solution; in the latter case the pK values determined would be different from pK_i depending on the ionic double layer potential (ψ_0). This fact was taken into account by multiplying $[H^+]$ in the first term of Eq. 4 by '$\exp(-z\times\psi_o\times F/R\times T)$', where ψ_o was calculated by the Gouy-Chapman [33] equation as detailed in Appendix A.

As was shown in previous reports [18,29],

$$\Delta pK_i = pK_i - pK_w \text{ and } \Delta pK_m = pK_m - pK_w$$

where ΔpK_i represents the free energy necessary to transfer the acid and its conjugated base from water towards the membrane, so ($\Delta pK_i - \Delta pK_m$) represents the free energy necessary to transfer H^+ from water towards the membrane. The difference between the 'intrinsic' pK_m in the membrane and interfacial pK_i is the logarithm of the proton's activity coefficient and represents the 'primary medium effect' of the proton and can be approximated by the activity coefficient of H^+ in HCl or NaOH. These values are expressed for each dioxane-water mixture as 'degenerate activity coefficients' (γ_0) [31].

Thus, if

$$\Delta pK_m - \Delta pK_i = \log \gamma_0$$

then

$$\Delta pK_i = \Delta pK_m - \log \gamma_0$$

2.7. Statistical analysis

Linear analysis was performed by the least-squares method. Comparisons were analysed by Student's *t*-test [34].

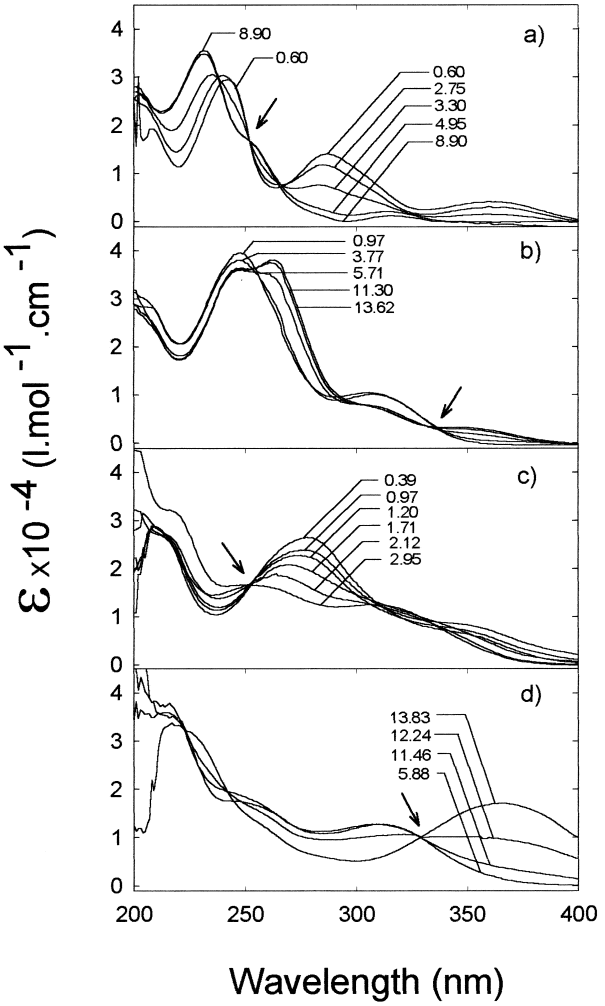

Fig. 2. Absorption spectra of benzodiazepines. UV absorption spectra of aqueous BZD solutions at different pH. (a) DZ (from pH = 0 to pH = 10); (b) CX (from pH = 0 to pH = 12); (c) CZ_1 (from pH = 0 to pH = 6); (d) CZ_2 (from pH = 6 to pH = 14). Arrows indicate the isosbestic points.

3. Results

Fig. 2 shows UV absorption spectra of aqueous solutions of BDZs at different pH. For each BZD, pH values were chosen to cover a wide range around the p*K*. In the particular case of CZ, which has two protonation sites, we show in separate graphs (Fig. 2c,d), the UV spectra obtained within pH ranges centred at the corresponding p*K* for each acid-base equilibrium reaction. It was evident that for every BZD an isosbestic point existed at the wavelength point where spectra cross up (DZ: 255 nm; CX: 335 nm; CZ_1: 256 nm; CZ_2: 330 nm), indicating the presence of only two (DZ and CX) or three (CZ) absorbing chemical species participating in one or two equilibrium reactions respectively. The isosbestic points were confirmed by a linear combination analysis as proposed by Nowicka-Jakowsika [35] (not shown).

Table 1
Effect of the environmental polarity on the BZD acid-base equilibrium in homogeneous media

BZD	[Diox.]	*D*	p*K*	ΔpK_m	log γ_0	ΔpK_i
DZ	0	78.36	2.92	–	0	0
	20	60.79	1.58	−1.34	0.15	−1.49
	45	38.48	1.16	−1.76	0.50	−2.26
	70	17.69	0.89	−2.03	1.30	−3.33
	82	9.53	0.71	−2.21	2.25	−4.46
CX	0	78.36	5.15	–	0	0
	20	60.79	3.63	−1.52	0.15	−1.67
	45	38.48	3.21	−1.94	0.50	−2.44
	70	17.69	2.77	−2.38	1.30	−3.68
	82	9.53	2.83	−2.32	2.25	−4.57
CZ_1	0	78.36	1.23	–	0	0
	20	60.79	−0.18	−1.41	0.15	−1.56
	45	38.48	−0.79	−2.02	0.50	−2.52
	70	17.69	−0.97	−2.20	1.30	−3.50
	82	9.53	–	–	2.25	–
CZ_2	0	78.36	10.09	–	0	0
	20	60.79	10.27	0.18	0.15	0.03
	45	38.48	10.45	0.36	0.50	−0.14
	70	17.69	–	–	1.30	–
	82	9.53	10.41	0.32	2.25	−1.93

[Diox.], dioxane concentration (% v/v). CZ_1 and CZ_2 correspond to the two equilibrium reactions for CZ. *D*, dielectric constant of the dioxane-water mixture [31]. At [Diox.] = 0, $pK = pK_w$, and at [Diox.] > 0, $pK = pK_m$; $\Delta pK_m = pK_m - pK_w$ represents p*K* in homogeneous media of different polarity; γ_0, proton degenerate activity coefficient; log γ_0, primary medium effect [31]. $\Delta pK_i = \Delta pK_m - \log \gamma_0$ (interfacial p*K*).

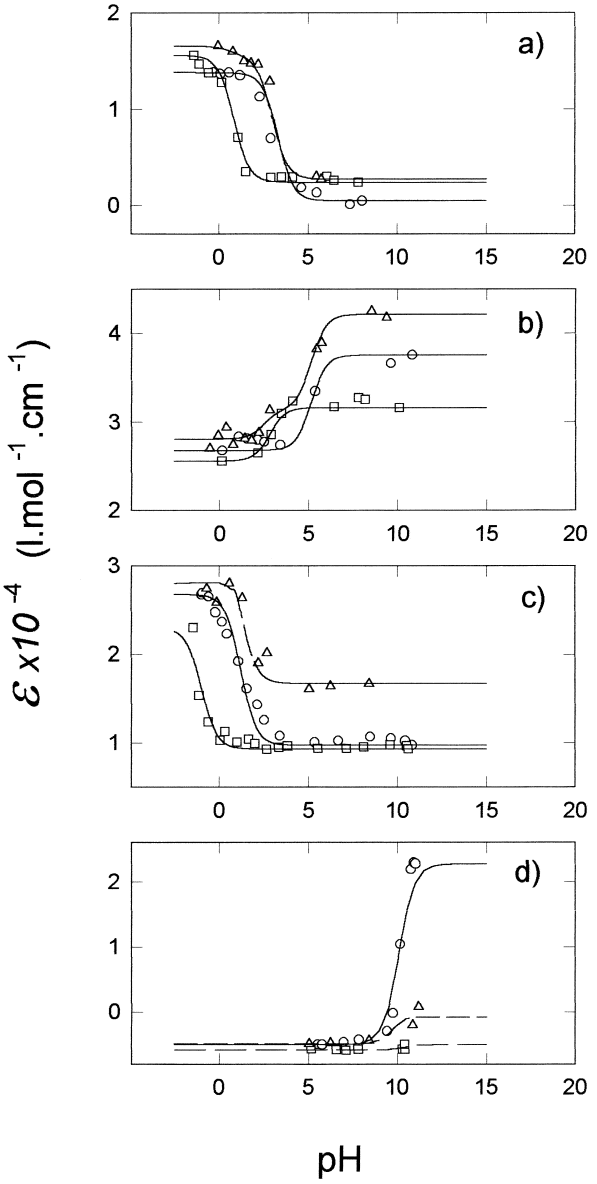

Fig. 3. Variation of benzodiazepine extinction coefficients as a function of corrected pH. UV extinction coefficients of different BZDs: (a) DZ; (b) CX; (c) CZ_1; (d) CZ_2 solubilized in water (○), in dioxane 82%, v/v or 70%, v/v (only in the case of CZ) (□) and in aqueous dispersions of Triton X-100 (△). Extinction coefficients were determined at the following wavelengths: DZ, 287 nm; CX, 264 nm; CZ_1, 280 nm; CZ_2, 355 nm. Lines correspond to theoretical curves calculated according to Eq. 3 or Eq. 4 as explained in Section 2.

In DZ spectra (Fig. 2a) the main peak increased its absorbance values and suffered an ipsochromic shift (from 240 nm to 230 nm) as the pH increased; conversely, for CX (Fig. 2b) the absorbance at 245 nm decreased and a shoulder at 265 nm appeared as pH increased. Fig. 2c and d show a decrease in the absorbance values and an ipsochromic shift (from 278 nm to 255 nm) in CZ spectra around the pK_1 and, for the second equilibrium, around a pH close to pK_2 the peak at 310 nm disappeared and a new peak at 370 nm was observed.

Fig. 3 shows the experimental values of observed absorbance (A_{obs}) vs. corrected pH for the different BZDs in water, in dioxane at high concentrations (70% or 82%, v/v) and in aqueous dispersions of Triton X-100. The theoretical fitting of absorbance values to Eq. 3 (for homogeneous systems) or to Eq. 4 (for Triton X-100 dispersions) showed a good adjustment of experimental data and allowed the calculation of pK values for the dissociation reactions (Section 2.5.

Table 1 shows the pK values of the different BZDs in water and in aqueous dioxane solutions calculated from Eq. 3 as explained above. In all cases, except pK_2 of CZ whose behaviour was opposite, the pK values decreased with decreasing polarity of the medium. ΔpK values (ΔpK_m and ΔpK_i) of different systems were calculated according to Section 2.6.

The values of the dielectric constants of the membrane region where the different BZDs are localised were estimated by interpolating, in ΔpK_i vs. D plots (Fig. 4), the ΔpK_i obtained in aqueous dispersions of Triton X-100 or in phospholipid-cholesterol mixtures (only for CX). This information is summarised in Tables 2 and 3. Electrostatic effects as a consequence of pH changes were absent when we used Triton X-100 micelles because they are neutral within the whole pH range tested. However, the zwitterionic

Table 2
Benzodiazepine pK_i and ΔpK_i values in Triton X-100 dispersions

	pK_w	$pK_{i\ Triton}$	$\Delta pK_{i\ Triton}$	D_{Triton}
Diazepam	2.92	0.30	−2.62	36
Chlordiazepoxide	5.15	2.50	−2.65	37
$Clonazepam_1$	1.23	0.16	−1.06	64
$Clonazepam_2$	10.09	10.17	0.08	62
Flunitrazepam[a]	1.52	0.89	−0.63	60

pK_w, pK measured in water; pK_i, interfacial pK values determined in aqueous Triton X-100 dispersions. $\Delta pK_i = p_iK_{i\ Triton} - pK_w$. D_{Triton}, dielectric constant of the localization region of BZDs within membrane, obtained by interpolation of $\Delta pK_{i\ Triton}$ values in the ΔpK_i vs. D curve corresponding to each BZD (see Fig. 4). [a]Taken from [18].

phosphatidylcholine vesicles used to test the effect of membrane composition on CX localisation are expected to be positively charged at $pH < pK_{phosphate}$ ($pK_{phosphate} < 1$ [36]) due to the presence of the trimethylammonium group at the choline moiety which is charged [36]. As the contribution of the negative charge from the ionisation of the phosphate group increases as a function pH, the whole net charge decreases and at $pH \geq 3$ it is close to zero. The surface potential (ψ_o) of PC liposomes changes according to surface effective charge density (σ_{eff}), so the latter magnitude will decrease as the cholesterol molar fraction increases in PC-cholesterol mixed liposomes and also as a function of pH as described above (see Appendix A for details).

The dielectric constant values of the membrane region where BZDs are localised obtained in aqueous Triton X-100 dispersions (D_{Triton}) for DZ ($D = 36$) and CX ($D = 37$) were smaller than those for CZ (CZ_1, $D = 64$ and CZ_2, $D = 62$) (Table 2).

The results shown in Table 3 obtained for CX in dispersions of mixed phospholipid-cholesterol MLVs indicate that as the cholesterol content increased from 0 to 20 mole%, the D values decreased from $D = 59$ to $D = 40$ in dpPC dispersions and increased from $D = 51$ to $D = 72$ in egg-PC dispersions.

4. Discussion

All 1,4-benzodiazepine-2-ones have one protonation site in the nitrogen atom at position 4. The ones containing a N-H bond at position 1 also have a second site of deprotonation [37]. The BZDs that participate in only one acid-base equilibrium exist as neutral molecules at pH over the pK and the ones that participate in a second acid-base equilibrium therefore exist as neutral molecules within the pH range $pK_1 < pH < pK_2$ (Fig. 1a,b). CX, a 4-*N*-oxide-1,4-benzodiazepine, has only one protonation site in the nitrogen atom at position 2. Too low pH values induce acid hydrolysis of CX to a

Table 3
Chlordiazepoxide pK_i and ΔpK_i values in phospholipid dispersions

	pK_i	ΔpK_i	D
dpPC	3.81	−1.34	59
dpPC (cholesterol 5%)	2.81	−2.34	44
dpPC (cholesterol 20%)	2.63	−2.52	40
egg-PC	3.29	−1.86	51
egg-PC (cholesterol 5%)	3.37	−1.78	54
egg-PC (cholesterol 20%)	4.58	−0.57	72

Phospholipids or phospholipid-cholesterol mixtures and dispersions were done as explained in Section 2, at a final lipid concentration of 1.5 mg/ml. Cholesterol content was calculated on a molar basis. D, dielectric constant obtained by interpolation of ΔpK_i values in ΔpK_i vs. D curves for CX (see Fig. 4).

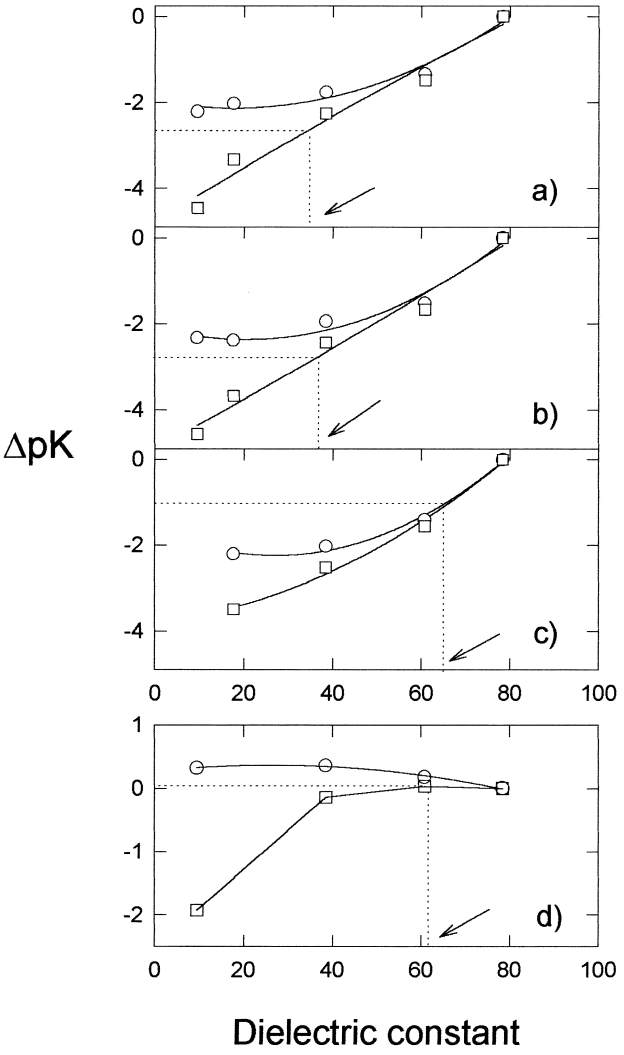

Fig. 4. Effect of the polarity of the medium on ΔpK values of some benzodiazepines. Points correspond to the ΔpK_m (○) and ΔpK_i (□) of BZDs in different aqueous dioxane solutions (0–82% dioxane v/v) shown in Table 1, vs. the corresponding dielectric constant values of the medium. Dotted lines indicate the interpolation of ΔpK_i of each BZD in Triton X-100. (a) DZ; (b) CX; (c) CZ_1; (d) CZ_2. Arrows point to D values of the membrane regions where the BZD is localized.

benzodiazepin-2-one-4-oxide before the oxygen atom at the *N*-oxide position could be protonated [38]. This fact implies that for CX it is possible to measure just one p*K*. The stability of DZ was unaffected, as checked by UV spectroscopy, after a treatment with 6 M H_2SO_4 for 15 min at 25°C either in the presence or in the absence of Triton X-100. The results obtained for CZ localisation were similar independently of the equilibrium reaction analysed, so the stability of this BZD at low pH was not investigated further.

Fig. 2 shows only one isosbestic point for DZ and CX UV spectra (Fig. 2a,b). This indicates the presence of two chemical species (protonated and non-protonated or neutral) which participate in the acid-base equilibrium reaction and can be encountered either together in equilibrium or alone at the extremes of the titration curve. In the case of CZ two isosbestic points can be identified (Fig. 2c,d) confirming the presence of the three chemical species participating in two acid-base equilibria: (a) protonated and neutral or (b) neutral and negatively charged, within pH ranges around the pK_1 and pK_2 respectively.

The tendency of BZDs to dissociate depended on polarity changes within their environment which could be evidenced by p*K* shifts (4; 1). The drop in p*K* observed while the polarity of the medium decreased (Fig. 4a–c), except in pK_2 of CZ (Fig. 4d), proves that dissociated (neutral) species are favoured by the rise in medium hydrophobicity. The opposite behaviour observed with the pK_2 of CZ demonstrated its tendency to keep bound the second proton in the N-H bond at position 1 as the polarity of the environment decreased.

The *D* values obtained for the region of BZD-membrane interaction in micelles of Triton X-100, or bilayers of dpPC or egg-PC (Tables 2 and 3) indicated that in addition to FNTZ [18,19,24] other BZDs were able to incorporate as an integral part of the bilayer; moreover, BZDs would be located neither within the membrane core ($D \approx 2$–5) as was suggested by fluorescence polarisation experiments [7,8] nor just be adsorbed at the surface ($D \approx 78$) as was suggested by binding experiments at high drug concentrations [16].

At the assayed temperature the dpPC liposomes were in gel phase while the egg-PC liposomes were in liquid-crystalline phase. The effects of cholesterol on phospholipid membrane are well known [39]. Cholesterol can induce different changes on viscosity and permeability of the membrane; it induces an increase in the membrane viscosity of liquid-crystalline phases restricting the mobility of the molecules, and a decrease in the viscosity of gel phases where the motion of hydrocarbon chains is already restricted [39]. In this context the *D* values obtained for the region where CX interacts with dpPC or egg-PC vesicles can be interpreted considering that this drug localises more profoundly as the bilayer becomes less viscous and vice versa. However, the presence of cholesterol also affects the hydration of the polar head region of PC bilayers [40], so both aspects should be taken into account in the interpretation of *D* values.

For PC in the L_α (liquid crystalline) phase, complete miscibility has been clearly established by small angle neutron scattering studies up to cholesterol molar fractions of $x_{chol} = 0.14$ and there was evidence that this was also the case up to $x_{chol} = 0.45$ [41]; the steady state anisotropies of anthroyloxy fatty acid probes in PC-cholesterol mixed MLVs above the T_c of the PC increased approximately linearly up to $x_{chol} = 0.5$ [42]; in addition a continuous increase in hydration was observed at least in the range of $0 < x_{chol} < 0.25$ [43]. The *D* values of CX accompanied this changes in the same sense (Table 3). The increase in *D* values may be interpreted as more external localisation of the drug in the membrane or it may indicate that the drug is just sensing a polarity change towards a higher hydrophilicity in the environment where it is located due to the enhancement of cholesterol mole fraction in the vesicles.

In the case of PC in the L_β (gel) phase, phase boundaries were clearly established at $x_{chol1} = 0.08$, $x_{chol2} = 0.024$ and $x_{chol3} = 0.435$. At $x_{chol} < 0.08$ there was full miscibility in a solid solution where hydrocarbon chains are tilt. At $0.08 < x_{chol} < 0.24$ it coexisted with a non-tilt mixture containing 24 mole% of cholesterol [41]. A phase boundary at $x_{chol} = 0.2$ has also clearly been observed for mixed monolayers of dpPC and cholesterol [44]. The number of water molecules bound to dimyristoyl-PC stays constant up to $x_{chol} = 0.4$ and then increases strongly until it seems to level off at $x_{chol} = 0.5$ [45]. So within the range of

x_{chol} used in our experiments, structural changes but not hydration changes are expected. Consequently, the more significant change in D value was observed at a x_{chol} close to that necessary for the phase boundary to appear and, at higher x_{chol}, D decreased only slightly. In this case it may be possible that CX can be accommodated more deeply in the interface as x_{chol} increases, perhaps due to the existence of defects in the structural arrangements of the bilayer.

Comparison between D values in Triton X-100 and in PCs is not so simple. Several experimental evidences indicate that the surface of Triton X-100 micelles is more polar than the surface of both lyso-PC (LPC) micelles and PC bilayer. From the apparent ΔpK shifts induced by cetyltrimethylammonium (CTAC) micelles on local anaesthetics, different values of the surface potential (ψ_o) of CTAC were calculated depending on whether the neutral Triton X-100 ($\psi_o = 41$ mV) or zwitterionic LPC micelles ($\psi_o = 77$ mV) were used to determine the intrinsic pK (pK_i) [46]. This discrepancy may be attributed to a higher ΔpK_i in Triton X-100 with respect to LPC. Taking into account that the ionisation reaction is favoured (pK_i decreases) in more polar environments, it can be concluded that these results reflect the higher polarity of the surface of Triton X-100 micelles compared to that of LPC micelles. On the other hand, apparent pK values of 6.7 and 7.2–7.6 were determined for the fatty acid carboxyl group in Triton X-100 micelles and in PC vesicles respectively, indicating that in those 'neutral' lipids the carboxyl group senses different microenvironments [47]. It has also been reported that the dielectric constants measured at the surface of micelles for various detergents are higher than those of phospholipid [48]. Moreover, fluorescent spectroscopic studies suggest that the microenvironment of the fluorescent probe in the surface of the detergent micelle is more polar than the phospholipid-water interface [49].

In addition to the difference in polarity, there are several structural differences between micelles and bilayers, like surface curvature and molecular packing. It is known that the surface area per molecule of a fluid phospholipid bilayer is 0.57 nm^2 as measured by X-ray diffraction [36]. On the other hand, from Stoke's ratio (4.1 nm) [50] and the mean aggregation number (143 molecules) [50], a surface area per molecule of 1.48 nm^2 can be calculated for Triton X-100 micelles suggesting that the molecular packing of Triton X-100 in micelles is significantly lower than that of PC in vesicles. Surface polarity is inversely related with molecular packing as shown by changes in the ionisation pK of hydroxycoumarin which increases from 9.0 to 10.2 in monolayers of dpPC upon decreasing the molecular area from 1.05 nm^2 (liquid expanded phase) to 0.43 nm^2 (solid condensed phase) [48] (note that the ionisation reaction is unfavoured (pK increases) in less polar media). BZDs have the tendency of partitioning from water to less polar media [6,32]. However, the ability of FNTZ to expand dpPC monolayers, when it penetrates from the subphase into the monolayer, increases as the lateral surface pressure decreases [24] in spite of the fact that low surface pressures are associated with low molecular packings and, consequently, with high polarities. Hence, the lower molecular packing in Triton X-100 micelles with respect to PC vesicles may be the factor that determines the localisation of CX deeper in the former with respect to the latter.

The D values in dpPC and those in egg-PC may reflect the result of the combination of two opposite effects: molecular order and hydration. In the absence of cholesterol $D_{dpPC} > D_{egg-PC}$ (Table 3). This may be the consequence of the higher order in gel phase compared with that of the liquid crystalline which is combined with the fact that hydration of a liquid-crystalline phase is higher than that of a gel phase [45] leading to a difference in D values which was rather small. Another reason for the small difference may be related with the location of BZD within the polar head group; it has been demonstrated that, in bilayers, there is a 'fluidity gradient' growing toward the methylene end of the hydrocarbon chains and in addition, when comparing the structural order in bilayers above and below the transition temperature, a small difference was observed at C_2 compared with C_{10} [51]. So, if the value of D was expected to depend on the environmental structural order, the location of BZDs within the polar head group region would predict a small difference between D_{dpPC} and D_{egg-PC}.

Data from EPR experiments indicate that the fatty acid polymethylene chain in aqueous dispersions of dpPC and egg-PC mixtures with cholesterol (2:1

mole ratio) may be thought of as relatively 'rigid rods' for a region of up to about eight carbon atoms from the fatty acid-glycerol ester linkage, with rapidly increasing probabilities for gauche states at larger distances [51]. In the presence of cholesterol, the combined effects of order and hydration favour the latter leading to D values in egg-PC-cholesterol MLVs higher than those in dpPC-cholesterol.

In conclusion, BZDs interact with membrane-water interfaces at different depths depending on the chemical structure of the drug (Table 2) and on membrane composition (Table 3) within the region of polar groups. As the membrane molecular order, molecular packing and hydration decrease, BZDs could locate more internally in this region.

These results should be taken into account when the pharmacological relevance of BZD active concentration is evaluated because the depth and extent of partitioning of these drugs into biomembranes would be coupled to local oscillations of membrane dynamics which may be induced by physiological events.

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Appendix

The surface potential for a flat surface can be calculated according to the equation of Guy-Chapman [33]:

$$\psi_0 = \frac{2\times k\times T}{e}\times \ln[x + (x^2+1)^{1/2}] \qquad \text{(A1)}$$

$$x = \sigma_{\text{eff}}/(8\times k\times T\times n\times \varepsilon_r\times \varepsilon_o)^{1/2} \qquad \text{(A2)}$$

where the symbols have the following meanings: e, elemental charge; k, Boltzman constant; T, absolute temperature; σ_{eff}, effective charge per unit area; n, bulk ion number in molecules per unit volume; ε_r, relative permittivity of the medium (water); ε_o, permittivity of free space.

The value of σ_{eff} will change as a function of surface composition, pH and electrolyte concentration [52]:

$$\sigma_{\text{eff}} = \frac{e\times(1-x_{\text{chol}})}{x_{\text{chol}}\times A_{\text{chol}} + (1-x_{\text{chol}})\times A_{\text{PC}}} + \frac{1-\dfrac{K}{K+10^{-\text{pH}}\times\exp(-z\times\psi_o F/(R\times T))}}{1+K_b\times[\text{NaCl}]\times\exp((-z\times\psi_o F/(R\times T))} \qquad \text{(A3)}$$

where x_{chol} is the molar fraction of cholesterol in phosphatidylcholine (PC)-cholesterol mixed liposomes; A_{chol} and A_{PC} are the molecular areas of cholesterol and PCs ($A_{\text{chol}} = 0.39$ nm^2 and $A_{\text{dpPC}} = 0.525$ nm^2 (data from Perillo and Maggio, unpublished) and $A_{\text{egg-PC}} = 0.7$ nm^2 [52]) at 35 mN/m which is the approximate equilibrium lateral surface pressure of a bilayer [53]; F is the Faraday constant; R is the gas constant; K_b is the binding affinity constant of the counterion ($K_b \cong 1$ [36]); K is the equilibrium dissociation constant of the phosphate group of PC at the interface ($K \cong 1$ [36]); [NaCl] and z are the molar concentration and valence of the electrolyte counterion.

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