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Localization of flunitrazepam in artificial membranes. A spectrophotometric study about the effect the polarity of the medium exerts on flunitrazepam acid-base equilibrium

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Abstract

In the present paper we tried to test the hypothesis that nonspecific flunitrazepam–membrane interactions are consistent with drug molecules accommodated between lipid molecules, becoming an integral part of the bilayer. We developed a spectrophotometric method to determine $FNTZH^+$ equilibrium dissociation constant and applied it to the study of the acid-base equilibria of this drug in homogeneous media of different polarity. In these conditions, pK decreased with the decrement in the dielectric constant (D) of the media. These results, analyzed under the light of the theory developed by Fernandez and Fromherz (1977; J. Phys. Chem. 81, 1755–1761) let us infer that flunitrazepam is localized a region with $D = 60$. This D value is lower that $D_{water} = 78$ and higher than D of hydrocarbon chains zone ($D = 2–5$) and would correspond to D of the region of polar groups. This result is compatible with the hypothesis.

Keywords: Flunitrazepam; Dipalmitoylphosphatidylcholine; Triton X-100; Polarity; Acid-base equilibrium; Spectrophotometry

1. Introduction

Benzodiazepines (BZDs), like flunitrazepam (FNTZ), are able to specifically bind to membrane proteins at thoroughly studied receptors [1,2], as well as to interact nonspecifically with the lipid part of the membrane [3]. In the present work we are concerned exclusively with the latter aspect.

Previous studies from our laboratory [3] let us demonstrate that membrane–BZD nonspecific interactions (measured at BZD concentration in the micromolar range) can be explained by a partition equilibrium model (unlimited incorporation of molecules) but not by Langmuir adsorption isotherms (limited number of binding sites) as had been suggested previously [4]. The application of the latter model appears to be justified for ligands which bind exclusively to the membrane surface. Under these conditions, the constant surface area limits the maximum amount of bound ligand. In contrast, the thermodynamic result of a partition equilibrium is consistent with drug molecules accommodated between lipid molecules, becoming an integral part of the bilayer inducing its swelling until it converts into a non-bilayer phase [5]. We have observed by transmission electron mi-

Abbreviations: BZD, benzodiazepines; FNTZ, flunitrazepam; dpPC, dipalmitoylphosphatidylcholine; c.m.c., critic micellar concentration; MLV, multillamelar vesicles

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croscopy that FNTZ at micromolar concentrations can induce a decrement in the diameter of dipalmitoylphosphatidylcholine (dpPC) vesicles; moreover, much lower FNTZ concentrations (within the nanomolar range) were able to widen dpPC's elution peak and to increase the proportion of nonexcluded vesicles from molecular filtration through Sephadex G-200, a result that can be interpreted on the basis of a decrease in vesicles mean size (García and Perillo, unpublished data). This results suggest that FNTZ might be able to induce a constant increment in lipid molecular areas as its concentration in the membrane (which is directly proportional to the bulk concentration) becomes higher; through this mechanism FNTZ may increase the curvature of the surface until it becomes incompatible with a bilayer phase. Following this rationale it can be inferred that when FNTZ interacts nonspecifically with membrane it becomes an integral part of the bilayer.

It has been argued that intrinsic pK values of membrane bound drugs are significatively different from those measured in bulk [6,7].

In the present paper, we developed a spectrophotometric method to determine FNTZ equilibrium dissociation constant and applied it to the study of the acid-base equilibria of this drug in media of different polarity; these results, analyzed under the light of theory developed by Fernandez and Fromherz [8] let us infer the localization of FNTZ in the membrane.

2. Materials and methods

2.1. UV absorption spectra

FNTZ solutions (15 nM final concentration) were prepared in dioxane–water mixtures containing increasing dioxane proportions (0%, 20%, 45%, 70% and 82% v/v) in order to obtain media with different polarities; pH values of each solution were adjusted between 0.1 and 4 by the addition of 37% p/p HCl, $\delta = 1.19$ g/ml and was measured with a pH meter (Orion Research 701A, sensitivity 0.001 pH units), equipped with a glass electrode.

FNTZ absorption spectra in each solution were measured against a blank of the same solvent and identical pH used for dissolving FNTZ. 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.

FNTZ spectrum was also determined in the presence of either Triton X-100 (3.5 mM final concentration; c.m.c.: 0.24 mM) or dpPC (1.36 mM final concentration; c.m.c.: 10^{-7} mM) dispersed in water. The pH values of these dispersions were adjusted as indicated above.

2.2. Preparation of dispersions

Phospholipid or detergent were dispersed in water above their c.m.c. by five cycles of 1 min heating at 70°C and 1 min vortexing. The dispersions were kept at room temperature until use (within 24 and 48 h of preparation). In these conditions dpPC molecules selfaggregate into multilamellar vesicles (MLV), and Triton X-100 molecules into micelles.

2.3. Correction of pH meter readings

Solutions with known proton stoichiometric concentrations were prepared in the same dioxane–water mixtures mention above, by the addition of HCl (37% p/p, $\delta = 1.19$ g/ml) and their pH values were calculated as $pH_{calc} = -\log([HCl])$ or measured with a pH meter 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 of the form:

$$y = ax^2 + bx + c$$

where $y = pH_{calc}$ and $x = pH_{obs}$, by nonlinear regressions 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 undistinguishable contributions of 'primary medium effect' and the 'liquid junction potential' from the pH readings [9].

2.4. Calculation of FNTZ equilibrium dissociation constant

Given the following chemical reaction:

$$FNTZ + H^+ \rightleftharpoons FNTZH^+ \quad (1)$$

C_0 being the total drug concentration:

$$C_0 = [\mathrm{FNTZH^+}] + [\mathrm{FNTZ}] \quad (2)$$

where the values between brackets correspond to molar concentrations of the associated ($\mathrm{FNTZH^+}$) and dissociated (FNTZ) forms of flunitrazepam, the equilibrium constant (K_{eq}) of reaction (1) has the form:

$$K = \frac{[\mathrm{FNTZH^+}]}{(C_0 - [\mathrm{FNTZH^+}]) \cdot [\mathrm{H^+}]} \quad (3)$$

The absorbance of flunitrazepam determined by UV spectrophotometry (A_{obs}) equals the sum of all the absorbing species so that: $A_{obs} = \Sigma A_{i,j}$ and $A_{i,j} = \epsilon_{i,j} \cdot b \cdot c_j$ where $A_{i,j}$ is the absorbance value of chemical species j at wavelength i (λ_i); $\epsilon_{i,j}$ are molar extinction coefficients of absorbent species j at λ_i; b is optical path (1 cm) and c molar concentrations of species j.

In the flunitrazepam solutions studied, where absorbance was read against a blank consisting of the proper solvent or dispersion:

$$A_{obs} = \epsilon_{\mathrm{FNTZH^+}} \cdot b \cdot [\mathrm{FNTZH^+}] + \epsilon_{\mathrm{FNTZ}} \cdot b \cdot [\mathrm{FNTZ}] \quad (4)$$

The values of $\epsilon_{\mathrm{FNTZH+}}$ and ϵ_{FNTZ} at λ_i can be calculated from the values of A_{obs}, at the same λ_i, in both ends of flunitrazepam titration curves as in extremely acid media ($\mathrm{pH} \ll \mathrm{p}K$) all flunitrazepam is in the form $\mathrm{FNTZH^+}$ ($[\mathrm{FNTZH^+}] = C_0$) while in extremely alkaline media ($\mathrm{pH} \gg \mathrm{p}K$) the predominant species is FNTZ (in this case $[\mathrm{FNTZ}] = C_0$). So:

$$\epsilon_{\mathrm{FNTZH^+}} = \frac{A_{obs1}}{C_0 \cdot b} \quad (5)$$

and

$$\epsilon_{\mathrm{FNTZ}} = \frac{A_{obs2}}{C_0 \cdot b} \quad (6)$$

Substituting each ϵ in Eq. (4) by Eq. (5) and Eq. (6), respectively; solving for [FNTZ] in Eq. (2) and substituting for this expression in Eq. (4):

$$A_{obs} = \frac{A_{obs1}}{C_0} \cdot [\mathrm{FNTZH^+}] + \frac{A_{obs2}}{C_0} \cdot (C_0 - [\mathrm{FNTZH^+}]) \quad (7)$$

Solving for $[\mathrm{FNTZH^+}]$ from Eq. (3) and replacing in Eq. (7)

$$A_{obs} = \frac{K \cdot [\mathrm{H^+}] \cdot A_{obs1} + A_{obs2}}{K \cdot [\mathrm{H^+}] + 1} \quad (8)$$

K can be calculated by measuring flunitrazepam absorbance as a function of pH and adjusting these experimental data to Eq. (8). Then $1/K = K_a$, this latter being the equilibrium dissociation constant from which $\mathrm{p}K$ values can be calculated as $\mathrm{p}K = -\log K_a$.

In heterogeneous systems like dispersion of lipids or detergents,

$$A_{obs} = A_m + A_w \quad (9)$$

where A_m and A_w are the absorbance values of the drug partitioned into the membrane and dissolved in water, respectively. The value A_m depends on the concentrations of the absorbing species and, the latter is a function of total drug concentration in the membrane (C_{0m}) so that:

$$C_{0m} = C_0 \cdot P \quad (10)$$

where P is the partition coefficient of the drug in the membrane–water system (we used self-aggregating structures from lipids or detergents as model membranes). Even though the partitioning tendency of the drug influences A_{obs} values, it is possible to derive an equation independent from P as well as from C_0 or C_{0m}, like Eq. (8).

Then, Eq. (9) results the sum of two expressions similar to Eq. (8).

$$A_{obs} = \frac{K_{app} \cdot [\mathrm{H^+}] \cdot A_{obs1m} + A_{obs2m}}{K_{app} \cdot [\mathrm{H^+}] + 1} + \frac{K_w \cdot [\mathrm{H^+}] \cdot A_{obs1w} + A_{obs2w}}{K_w \cdot [\mathrm{H^+}] \cdot + 1} \quad (11)$$

where

$$A_{obs1m} = A_{obs1} - A_{obs1w}$$

and

$$A_{obs2m} = A_{obs2} - A_{obs2w}$$

A_{obs1} and A_{obs2} are experimental values of absorbance measure in the heterogeneous system at the extremes of the titration curve. A_{obs1w} and A_{obs2w} are

species FNTZ and $FNTZH^+$ absorbance values in water measured in a separate experiment.

The values A_{obs1w} and A_{obs2w} can be corrected for drug partitioning into membrane using actual values of concentration in water which depend on individual partition coefficients of protonated and nonprotonated species (P_i), but they do not depend on individual values of mass or concentration as the absorbance values of interest are located at the extremes of the pH scale where only one of the species is present:

$$C_{iw} = \frac{m_0 + C_0 \cdot V_0}{V_0 + P_i \cdot V_m} \quad (12)$$

Finally, 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. (11).

3. Results

Fig. 1 shows FNTZ absorption spectra in media of different pH values (Fig. 1a) and different polarity (Fig. 1b and c). Higher pH values induced an increase in the intensity of the peak at $\lambda_{max} = 220$ nm; the peak at $\lambda_{max} = 279$ nm (pH ≈ 0.6) decreased in intensity and followed an ipsochromic shift from $\lambda_{max} = 279$ nm at pH 0.6 ($\epsilon_{max} = 25\,800$) to $\lambda_{max} = 252$ nm at pH 4.7 ($\epsilon_{max} = 19\,067$). The different spectra lines crosses within a 2 nm wavelength interval around 255 nm and at a value of extinction coefficient of 1.88 ± 0.01 (0.63%). The dispersion in wavelength as well as in ϵ are within the experimental errors so this can be considered an isosbestic point. This fact was confirm by the linear combination analysis proposed by Nowicka-Jankowsika [10] (not shown). So, there is a reasonable evidence that only two absorbing species are present in the system under study.

The spectra shown in Fig. 1b and c were recorded in media of increasing dioxane concentration (to decrease the polarity) and at pH values at least two units above (Fig. 1b) or below (Fig. 1c) the dissociation constant of flunitrazepam in the corresponding media, so that the nonassociated form of FNTZ (Fig. 1b) or the protonated form (Fig. 1c) were the predominant species in solution. As the dielectric constant of the media decreased, the spectra showed a bleaching

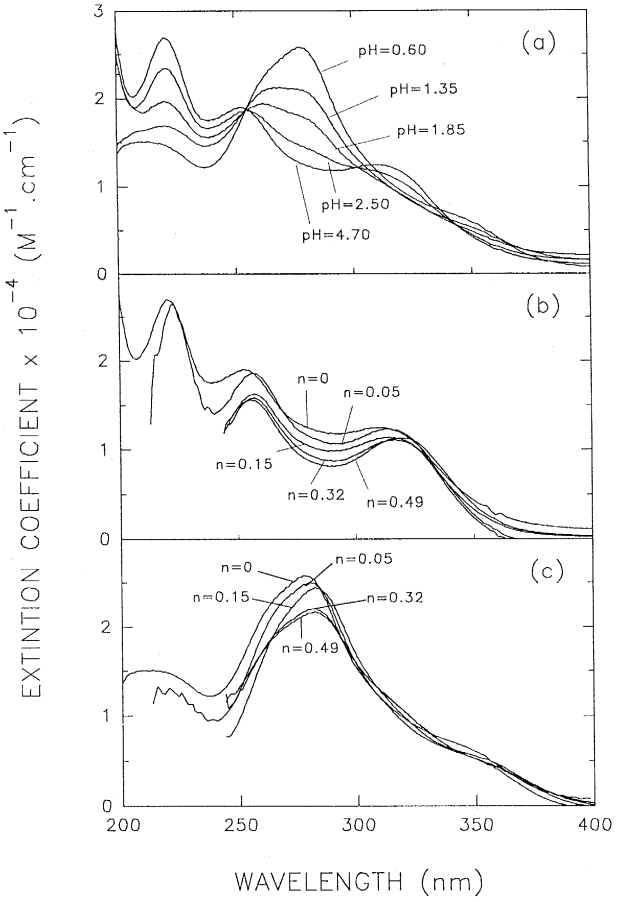

Fig. 1. Absorption spectra of flunitrazepam. (a) UV absorption spectra of aqueous flunitrazepam solutions in media of different pH (between 0.6 and 4.7). (b) Flunitrazepam was dissolved in different dioxane–water mixtures; n = dioxane molar fraction at pH over pK of flunitrazepam. (c) Same as in b except that pH was below pK.

effect and a batochromic shift of the peaks at $\lambda_{max} = 220$ nm, 252 nm and 310 nm (Fig. 1). Similar effects were observed respect to the peak at $\lambda_{max} = 279$ nm corresponding to $FNTZH^+$ in Fig. 1c.

Fig. 2 shows plots of pH_{calc} (stoichiometric pH) vs. pH_{obs} (readings from the pH meter) of HCl solutions in different dioxane–water mixtures. Although low dioxane containing mixtures showed a behavior very closed to that of water, dioxane concentrations above 45% v/v induced significant deviations from water. These plots were well fitted to second degree polynomials; the results of the regression analysis by the least squares method is shown in

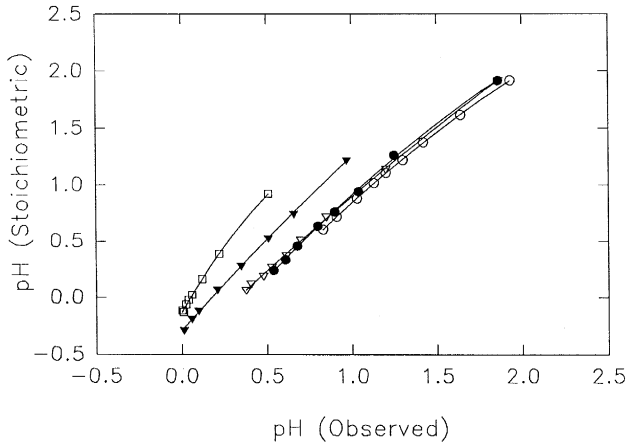

Fig. 2. pH correction curves. pH stoichiometric was calculated as −log([HCl]); pH observed: correspond to readings from the pHmeter. Dioxane molar fractions in dioxane–water mixtures: 0 (○), 0.05 (●), 0.15 (▽), 0.32 (▼), 0.49 (□). The lines were obtain by adjusting the experimental points to second degree equations by the least squares method. See Table 1 for analysis.

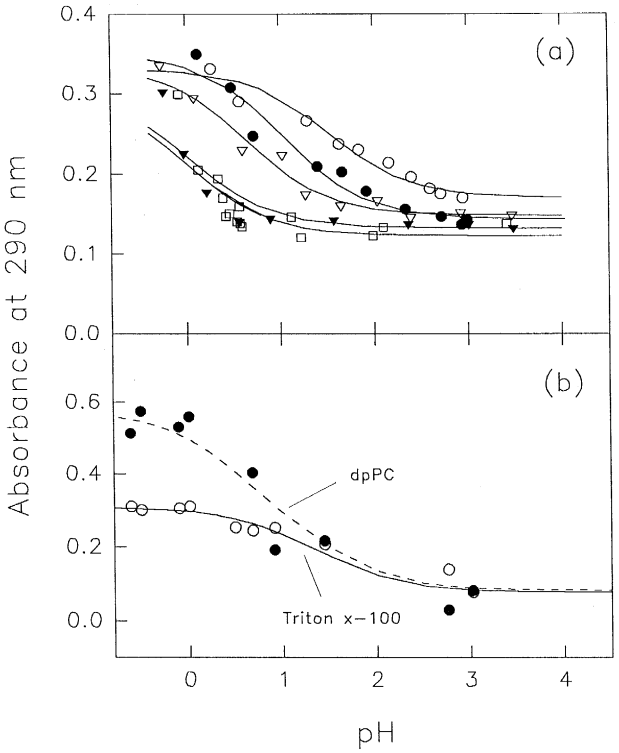

Fig. 3. Variation of flunitrazepam absorbance as a function of pH in media of different polarities. (a) UV spectra of flunitrazepam solubilized in dioxane–water homogeneous mixtures with the following dioxane molar fractions: 0 (○), 0.05 (●), 0.15 (▽), 0.32 (▼), 0.49 (□). (b) Flunitrazepam spectra in heterogeneous aqueous dispersions of Triton X-100 (○), or dpPC (●). Lines correspond to theoretical curves calculated with Eq. (8) (a), or Eq. (11) (b) (see Section 2).

Table 1. These equations were used to correct pH readings in other experiments.

Experimental values of observed absorbance (A_{obs} at 290 nm) were plotted as a function of corrected pH values (pH_{calc}; these measurements were done in solutions of varied dioxane concentration between 0% and 82% v/v (Fig. 3a) and in water dispersions of Triton X-100 or dpPC (Fig. 3b). In the figures, experimental points and their fitting to Eq. (8) (Fig. 3a) or to Eq. (11) (Fig. 3b), are shown. In the homogeneous systems of dioxane–water mixtures A_{obs} values decreased with increasing pH and they also decreased with decreasing polarity of the medium. By adjusting these data to Eq. (8) and applying the transformations indicated in Section 2, pK_a values for the equilibrium dissociation constants of flunitrazepam were calculated. These pK_a shifted

Table 1
Regression analysis of the plots for correcting pHmeter readings

Dioxane (% v/v)	x_{diox}	D	a_0	a_1	a_2	r	gl
0	0	78.36	-0.715 ± 0.023	1.766 ± 0.035	-0.208 ± 0.013	0.999	7
20	0.050	60.79	-0.677 ± 0.048	1.814 ± 0.092	-0.224 ± 0.038	0.999	6
45	0.147	38.48	-0.473 ± 0.024	1.471 ± 0.055	-0.099 ± 0.024	0.999	7
70	0.320	17.69	-0.289 ± 0.010	1.682 ± 0.056	-0.136 ± 0.057	0.999	7
82	0.490	9.53	-0.130 ± 0.007	2.598 ± 0.107	-0.105 ± 0.203	0.999	6

Plots of stoichiometric pH vs. observed pH (Fig. 2) were adjusted to second degree equations. a_x, coefficients (the subscript indicates the exponent of the independent variable); r, correlation coefficient. x_{diox}, dioxane molar fraction. The values of coefficients ± S.E.M. are shown. gl, degrees of freedom.

Table 2
Flunitrazepam pK and ΔpK values in dioxane–water mixtures

Dioxane (%)	x_{diox}	D	pK_a	ΔpK_m	$\lg\gamma_0$	ΔpK_i
0	0	78.36	1.523	0.000	0.00	0.000
20	0.050	60.79	1.036	−0.487	0.15	−0.637
45	0.147	38.48	0.646	−0.877	0.50	−1.377
70	0.320	17.69	0.336	−1.187	1.30	−2.487
82	0.490	9.53	0.215	−1.308	2.25	−3.558

x_{diox}, dioxane molar fraction. $\Delta pK_m = pK_a - pK_{a,\,water}$. $\lg\gamma_0$, primary medium effect. $\Delta pK_i = \Delta pK_m - \lg\gamma_0$. γ_0, 'degenerate activity coefficient' of the proton (data from Ref. [9]).

to lower values with the increase in dielectric constant (higher water content, higher polarity) (Table 2). Following the nomenclature used in Appendix A, pK_a and values correspond to pK_w (for solutions of flunitrazepam in pure water) or pK_m (for solutions of flunitrazepam in dioxane–water mixtures). ΔpK_m values were calculated, according to Eq. (A2), as the difference of pK_m in a dioxane containing mixture respect to the value in pure water (K_w); ΔpK_i values, were calculated according to Eq. (A3) by using values of degenerated activity coefficients of HCl from Van Uitert and Haas [9] (Table 2). The values of pK_{app} calculated from Eq. (11) in heterogeneous media containing Triton X-100 or dpPC are pK_i values because $[H^+]$ concentration used was that in bulk and not the one in the artificial membrane. The results obtain were: $pK_{i\ Triton} = 0.894$ and $pK_{i\ dpPC} = 0.537$.

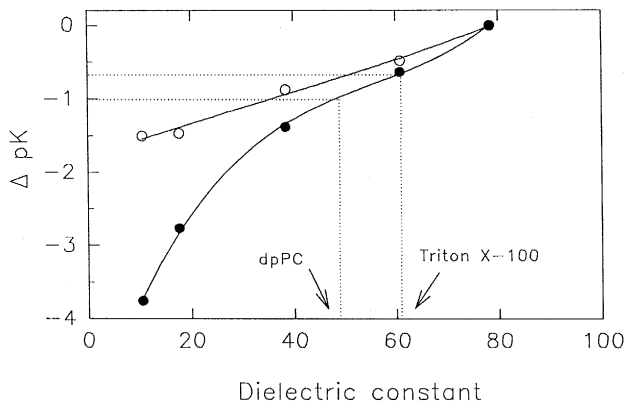

Fig. 4. Effect of dielectric constant on ΔpK values. Points correspond to ΔpK_m (○) and ΔpK_i (●) of FNTZ in dioxane–water mixtures of varied dioxane content. D, dielectric constant of the dioxane solution (taken from Ref. [8]). Arrows indicate the value of dielectric constant corresponding to the interpolation of ΔpK_i of flunitrazepam in Triton X-100 or in dpPC.

Fig. 4 shows plots of ΔpK_m and ΔpK_i as a function of the dielectric constant (D) of each dioxane–water mixture (see Table 2). From pK_i of flunitrazepam in Triton X-100 and in dpPC (Fig. 3b) and the value of pK_w (Table 2), $\Delta pK_{i\ Triton}$ (−0.629) and $\Delta pK_{i\ dpPC}$ (−0.986) values were calculated. These values were interpolated into the plot of ΔpK_i vs. D (Fig. 4) and approximated D values of the membrane region where flunitrazepam is interacting (in Triton X-100 $D = 60$, in dpPC $D = 50$) were determined. The discrepancies observed in the values of D between Triton X-100 and dpPC may be due to the fact that while the former exhibited a neutral interface in the whole range of pH studied, the latter ($pK < 1$) was positively charged in part of that pH range. For that reason, what is actually measure in dpPC is pK_{mw}; this pK has a dielectric (ΔpK_i) as well as an electrostatic component ($-F \cdot \Psi/2{,}3\ RT = -0.357$); from the electrostatic component we calculated an interface electric potential of +21 mV which may correspond to the mean electrical potential of the interface within the studied pH range.

4. Discussion

Over a range of low pH values flunitrazepam is positively charged due to its protonation of the nitrogen atom at position 4 [11]. This protonation induces changes in flunitrazepam spectra as a function of pH (the spectra of $FNTZH^+$ and FNTZ are different, Fig. 1) and has implications on drug partitioning in heterogeneous media (see Eq. (10), Eq. (12)) because of the differential preference that both species exhibit towards water and membrane. On the other hand, differences in extinction coefficients of charged and

uncharged species were also observed, due to changes in the polarity of the media (Fig. 1b and c). All these facts will affect the values of A_{obs1w} and A_{obs2w} (taken from the spectra of flunitrazepam in aqueous solutions set at pH far over or bellow pK_w) which should not be used in Eq. (11) without correction for drug partitioning. However, this precaution may not be necessary under experimental conditions like the presents, of relatively low P values of flunitrazepam ($P_{FNTZ} = 24$, Ref. [12]) and a membrane volume (V_m) negligible compared with aqueous volume (V_w) due to a relatively low lipid concentration. For these reasons, we could approximate [FNTZ] in the aqueous phase of the heterogeneous media to C_0. This introduced an error of about 3% in [FNTZ] (C_{2w}) as well as in A_{obs2w} (Eq. (12)); due to the lower partition coefficient of the charged species,the error in [$FNTZH^+$] (C_{1w}) and in A_{obs1w} was even lower. The fitting to Eq. (11) of the experimental values of A_{obs} in aqueous dispersions of Triton X-100 or dpPC was good (Fig. 3b), and no difference was observed with or without the corrections indicated above. So, low membrane volumes and low partition coefficients allow the determination of pK_m without the knowledge of individual P for charged and uncharged forms of the drug, which represents a practical advantage of the present method respect to others that appear in the literature [13].

The value of $D = 60$ calculated for the dielectric constant of the interface region where flunitrazepam interacts with a Triton X-100 neutral interface differs not only from that of water ($D = 78$) but also from that of the hydrocarbon chain core zone ($D = 2–5$) [14], so it follows that flunitrazepam interacts with the membrane–water interface at the region of polar groups. This result is compatible with the hypothesis that when flunitrazepam interacts with membrane it becomes part of the bilayer. To our knowledge, except from ours, the few studies that have been done with 1,4-benzodiazepines (BZDs) in relation with nonspecific drug–membrane interactions were fluorescence polarization studies using 1,6-diphenyl-1,3,5-hexatriene (DPH) as the fluorescent probe [15,16]. These studies showed that the BZD molecule was capable of inducing a decrease in membrane order. Taking into account that DPH is a highly lipophilic substance that dissolves in the hydrocarbon chains region of the membrane, the fact that BZDs are able to affect DPH's fluorescence might induce the linear conclusion that BZDs is also located within the membrane core. As shown in the present paper, at least for flunitrazepam, this is not true.

According to Israelachvilli, the limiting models of the self-aggregating structure that an amphiphatic molecule can form, besides entropic restrictions, depends on geometric constraints imposed by the sphere and the plane to accommodate inverted conical, cylindrical or conical shaped molecules to form inverted micelles (or other inverted structure like hexagonal II), bilayers or micelles, respectively, as the relative size of the polar head group increases [17–19]. This corresponds to an increase in the surface curvature of the structure. If the particles considered are vesicles and the changes are moderated, what is expected is a stepwise reduction in their diameter. The results shown in the present paper strongly suggest that flunitrazepam, by expanding the polar region of the interface, may force the structure surface towards a more curved one and lead to the reduction in vesicle size we observed previously (García and Perillo, unpublished data).

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Appendix A. Changes in pK values induced by polarity in selfaggregating structures of amphipathic molecules

Theory and methods used were essentially those developed by Fernandez and Fromherz [8]. The titration of a drug in a media of known polarity allows to determine K 'intrinsic' values (correspond to pK_m in dioxane containing mixtures 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 medium of all chemical species participating in the equilibrium (in the present case: FNTZ, $FNTZH^+$ and H^+).

On the other hand, K_{app} 'apparent' values determined in and heterogeneous system as described

previously (Eq. (11)) are 'interfacial' pK_i [1] because the concentration of H^+ used for its calculation is that in water and not that in the membrane. If the interface is electrically charged, protons activity at the interface is different from that in the bulk of solution; in this case, values determined according to Eq. (11) would be pK_{mw}, whose difference respect to pK_i depends on the existence of membrane potential. (Ψ). As a consequence:

$$pK_w = -\log \frac{a^{w_{FNTZ}}}{a^{w_{FNTZH^+}}} \cdot a^{w_{H^+}}\ pK_i$$

$$= -\log \frac{f^{m_{FNTZ}} \cdot c^{m_{FNTZ}}}{f^{m_{FNTZH^+}} \cdot c^{m_{FNTZH^+}}} \cdot a^{i_{H^+}}$$

$$pK_m = -\log \frac{a^{m_{FNTZ}}}{a^{m_{FNTZH^+}}} \cdot a^{w_{H^+}}\ pK^{mw}$$

$$= -\log \frac{f^{m_{FNTZ}} \cdot c^{m_{FNTZ}}}{f^{m_{FNTZH^+}} \cdot c^{m_{FNTZH^+}}} \cdot a^{w_{H^+}}$$

where a_i are the activities and f_i activity coefficients so that $c_i = f_i \cdot a_i$.

Expressing these pK values as a function of the chemical potentials (μ_i), universal gas constant (R) and absolute temperature (T):

$$pK_w = (\mu^{ow}_{H^+} + \mu^{ow}_{FNTZ} - \mu^{ow}_{FNTZH^+})/2.3\,RT$$

$$pK_i = (\mu^{ow}_{H^+} + \mu^{om}_{FNTZ} - \mu^{om}_{FNTZH^+})/2.3\,RT$$

$$pK_m = (\mu^{om}_{H^+} + \mu^{om}_{FNTZ} - \mu^{om}_{FNTZH^+})/2.3\,RT$$

$$pK_{mw} = [(\mu^{ow}_{H^+} + \mu^{om}_{FNTZ} - \mu^{om}_{FNTZH^+}) - (F \cdot \Psi)]/2.3\,RT$$

If:

$$\Delta pK_i = pK_i - pK_w \quad (A1)$$

$$\Delta pK_i = [(\mu^{om}_{FNTZ} - \mu^{ow}_{FNTZ})/2.3\,RT] - [(\mu^{om}_{FNTZH^+} - \mu^{ow}_{FNTZH^+})/2.3\,RT]$$

ΔpK_i represents the free energy necessary to transfer the acid and its conjugated base form water towards the membrane.

$$\Delta pK_m = pK_m - pK_w \quad (A2)$$

$$\Delta pK_i - \Delta pK_m = -(\mu^{om}_{H^+} - \mu^{ow}_{H^+})/2.3\,RT$$

$(\Delta pK_i - \Delta pK_m)$ represents the free energy to transfer H^+ from water towards the membrane.

As $\mu^{om}_{H^+} - \mu^{ow}_{H^+} = 2.3\,RT \log f^0_{H^+}$, it follows that the difference between 'intrinsic' pK_m in the membrane and interfacial pK_i is the logarithm of protons' activity coefficient and represents the 'primary medium effect' of the proton and can be approximated by the activity coefficient of HCl.

These values are expressed for each dioxane–water mixture as 'degenerate activity coefficients'.

$$\Delta pK_m - \Delta pK_i = \log f^0_{H^+} \quad (A3)$$

$$\approx \log f^0_{HCl}$$

The study of acid-base equilibrium carried out in charged interfaces permits the determination of membrane electrical potentials as:

$$\Delta pK_{mw} = \Delta pK_i - (F \cdot \Psi/2.3\,RT) \quad (A4)$$

In this case, the total shift of pK in the heterogeneous medium from pK in water, is partitioned into a pK shift and into an electrostatic potential contribution which can be considered as a shift of 'local pH' derivable from the potential Ψ.

[1] Actually, pK_{app} and pK_i differ by a factor that measures the difference between stoichiometric concentrations and thermodynamic activities of chemical species within the membrane: log $(f^m_{FNTZ} / f^m_{FNTZH^+})$. However, if a neutral membrane is used as a reference state and, in addition, drug concentration is low, this correction is not needed.

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