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Study of partition of nitrazepam in bile salt micelles and the role of lecithin

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Abstract

The effect of trihydroxy (sodium cholate and sodium glycocholate) and dihydroxy (sodium deoxycholate and sodium glycodeoxycholate) bile salt micelles on the spectrophotometric properties and on the solubility of nitrazepam in aqueous solution, at 25.0°C and at ionic strength 0.1 M in sodium chloride, has been assessed. From the results obtained it was possible to calculate the partition coefficients (K_p) of nitrazepam between aqueous and micellar phases. The partition coefficients of nitrazepam have also been determined in mixed micelles of cholate or deoxycholate with lecithin (egg yolk phosphatidylcholine), which were used as a model of the gastrointestinal tract. Drug partition was found to depend on the bile acid (number of hydroxyl groups and conjugation with glycine), and our data indicate further that addition of lecithin to bile salt micelles decreases the values of the partition coefficients in the mixed micelles at physiological pH.

Keywords: Partition coefficients; Bile salt micelles; Solubility; Mixed micelles; Lecithin; nitrazepam

1. Introduction

Bile salts, having a large, rigid, and planar hydrophobic moiety of a steroid nucleus with two or three hydroxyl groups, are a special group of biosurfactants, whose properties differ considerably from ordinary aliphatic surfactant molecules [1,2], namely bile salts have lower aggregation number and rigidity, and smaller surface charge density than conventional aliphatic surfactants [3].

Micellar solubilization, the enhanced aqueous solubility of otherwise poorly soluble organic substances brought about by the presence of surfactant micelles, which is caused by their incorporation into micelles, plays a very important role in biological processes, such as adsorption and transfer of materials in living tissues [4,5]. Bile salts are considered to enhance the intestinal absorption of drugs orally administrated by interacting with the drug, eventually by drug

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incorporation after micelle formation, and also by altering the permeability of the biological membrane [6–10].

Bile salts in the human body are often associated with long-chain fatty acid esters of phosphatidylcholine in mixed micelles, and the physicochemical behavior of these mixed micelles has been extensively studied due to the potential applications in the design of drug-delivery systems for poorly soluble drugs and in oral formulations [11]. These mixtures have been used as bile models and the principles governing their self-assembly may help to understand the structure and action of native bile in emulsifying lipophilic compounds in the bile duct, gall bladder and intestine. In the recent 'locally cylindrical' model of mixed micelles [12], the acyl chains of phosphatidylcholine are oriented toward the center of the wormlike mixed micelle with their zwitterionic head groups facing outward. On the other hand, bile salt molecules both 'cap' the hydrophobic ends of the mixed micelle and are dispersed randomly on the surface of the micelles with their hydrophobic surfaces interacting with acyl chains of phosphatidylcholine and their hydrophilic surfaces and ionic side chains oriented toward the aqueous continuum [12]. The charge of the phospholipid is partially compensated by the hydroxylic groups of the bile salts, and as consequence the net superficial charge is smaller than in simple bile salt micelles, a factor that may affect the solubilizing capacity of the mixed systems [13]. Indeed, these mixed systems incorporate hydrophobic compounds due to their large size and the fluidity of their core, but drugs, which interact predominantly with the micellar electrostatic surface (opposite charges), are poorly incorporated in mixed micelles of bile salts/lecithin [13].

Quantification of the effect of micelles on spectroscopic properties and solubility of pharmaceutical drugs allows for the determination of partition coefficients. These changes reflect modifications in the microenvironment of the molecule undergoing solubilization and can provide a detailed picture for partitioning of the drug in micelles [14].

In this work we have quantified the partition coefficients of nitrazepam (1,3-dihydro-7-nitro-5-phenyl-1,4-benzodiazepin-2-one), a benzodiazepine, in aqueous solutions of bile salt micelles and assessed the role of added phosphatidylcholine in their values. We have determined the partition coefficients between the aqueous and micellar pseudo-phases by spectrophotometry and by solubility measurements, and in the latter case the data is compared with those obtained in SDS. The results have shown that drug partition depends on the bile acid (number of hydroxyl groups and conjugation with glycine). Furthermore, our data indicate that addition of lecithin to bile salt micelles decreases the values of the partition coefficients in the mixed micelles at physiological pH.

2. Experimental

2.1. Reagents and solutions

Nitrazepam, bile salts (sodium cholate, sodium glycocholate, sodium deoxycholate and sodium glycodeoxycholate), sodium dodecyl sulphate (SDS) and egg yolk lecithin (EPC) were from Sigma and used as received; all other chemicals were from Merck (pro analysi). All solutions were prepared with double deionized water (conductivity less than 0.1 $\mu S\ cm^{-1}$). Bile salt solutions were prepared by rigorous dilution of a stock solution of known titre, which has been determined by conductimetric titrimetry with HCl 0.1 M (Merck, Titrisol). For all solutions studied, the ionic strength (I) was adjusted to 0.1 M with NaCl.

2.2. pH and spectrophotometric measurements

The automatic system used to measure pH has been described elsewhere [15]; and system calibration was performed by the Gran method [16] in terms of hydrogen ion concentration, using strong acid/strong base titrimetry. All absorption spectra were recorded with a Hitachi U-2000 dual-beam spectrophotometer using quartz cells with 1-cm path length that were thermostated at 25.0°C.

In the solubility studies, calibration curves (A vs. concentration) were constructed for the neutral (308 nm) and for the deprotonated (364 nm)

forms of nitrazepam for each concentration of bile salts used. The Beer–Lambert law was obeyed in the concentration range used (1.0×10^{-5}–2.5×10^{-5} M for the neutral form, and 2.6×10^{-5}–5.0×10^{-5} M for the deprotonated form), both in aqueous and in micellar solutions. The reported data are the average of four different measurements, in which the blank was a solution with the same bile salt concentration and at the same ionic strength, but without the drug.

2.3. Solubility studies

The solubility of the neutral and deprotonated forms of nitrazepam was measured in water, and in aqueous SDS and in bile salt solutions, below and above the cmc. The following concentrations were used: for SDS, seven solutions in the range 1.0×10^{-3}–2.0×10^{-2} M; for sodium deoxycholate and sodium glycodeoxycholate, nine solutions in the range 1.0×10^{-3}–6.5×10^{-3} M; for sodium glycocholate, eight solution in the range 6.0×10^{-3}–4.5×10^{-2} M; for sodium cholate, nine solution in the range 4.0×10^{-3}–3.0×10^{-2} M. The cmc values for the bile salts in aqueous solutions with $I = 0.1$ M (NaCl) are: 6.0×10^{-3} M (sodium cholate); 1.2×10^{-2} M (sodium glycocholate); 2.5×10^{-3} M (sodium deoxycholate); and 2.2×10^{-3} M (sodium glycodeoxycholate) [17].

For each solution, the benzodiazepine was dispersed in 20 ml of each solvent and the pH adjusted to 7.0 or to 11.5, by addition of concentrated HCl or NaOH. The saturated solutions were incubated for 48 h at 25.0 ± 0.1°C in a thermostated bath, and then filtrated through filter paper (Lida, 0.45 μm). The concentration of nitrazepam was determined spectrophotometrically at the wavelength of maximum absorbance, after the appropriate dilution with a solution with the same composition, but in which the drug was absent. In each case, the corresponding solvent system diluted in the same way as the measured filtrate was used as to correct any absorbance of the surfactant and as a solvent to prepare working standard solutions of each drug studied for the construction of a calibration curve.

2.4. Preparation of mixed micelle solutions

Mixed micelles of lecithin (concentrations of 500×10^{-6} and 750×10^{-6} M) and bile salts were prepared by dissolving a known amount of lecithin in chloroform/methanol (10:1, v:v), to which was added sodium cholate or sodium deoxycholate by rigorous dilution of a concentrated methanolic solution. The mixture was dried under vacuum in a rotative evaporator, and the resulting film was dried under a stream of nitrogen in the dark for at least 1 h, and was then suspended in an aqueous solution ($I = 0.1$ M NaCl), using a vortex. For bile salt concentrations below the cmc, the product was a suspension of lecithin vesicles, whereas for concentrations higher than the cmc, clear solutions of mixed micelles were obtained. In the resulting solutions/suspensions, the bile salts were assayed by conductimetry, as described above, and the total concentration of lecithin was determined by an enzymatic method, using a kit obtained from Biomérieux (Lyon, France). The cmc values of the mixed bile salt/lecithin micelle in aqueous solutions with $I = 0.1$ M are: 9.0×10^{-4} M, for cholate and 750×10^{-6} M lecithin; 2.0×10^{-3} M for cholate and 500×10^{-6} M lecithin; 8.0×10^{-4} M, for deoxycholate and 750×10^{-6} M lecithin; and 1.2×10^{-3} M for deoxycholate and 500×10^{-6} M lecithin [17].

2.5. Spectrophotometric determination of partition coefficients

Aqueous solutions of nitrazepam were added to solutions of the desired micelles (either simple or mixed), and the resulting solutions were then incubated in the dark and at 25°C for 12 h. The partition coefficients of nitrazepam were calculated at pH 11.5 and 7.0.

In the determinations of partition coefficients in simple micelles, the concentrations of bile acid salts were: cholate between 1.0×10^{-4} and 4.0×10^{-2} M; glycocholate between 8.0×10^{-3} and 5.0×10^{-2} M; deoxycholate between 5.0×10^{-4} and 6.0×10^{-3} M; glycodeoxycholate between 5.0×10^{-4} and 6.5×10^{-3} M. For mixed micelles the following concentrations of surfactants were used: (a) 750×10^{-6} M lecithin, cholate from

5.0×10^{-4} to 3.0×10^{-2} M, and deoxycholate from 1.0×10^{-4} to 7.0×10^{-3} M; (b) 500×10^{-6} M lecithin, cholate from 4.0×10^{-4} to 3.0×10^{-2} M, and deoxycholate from 5.0×10^{-4} to 7.0×10^{-3} M. Total concentration of nitrazepam in the final solutions was always smaller than 5×10^{-5} M, and the blank was the same solvent system with the same concentration of surfactants, but without the drug.

3. Results

Nitrazepam has two acid equilibria in solution

$$H_2B^+ \rightleftharpoons H^+ + HB \rightleftharpoons H^+ + B^- \quad (1)$$

but in the pH range used (7 and 11.5) only the second equilibrium is important, as the pK_a values are $pK_{a1} = 2.98 \pm 0.01$ and $pK_{a2} = 10.55 \pm 0.03$ [15], and thus only two forms of the drug exist in significant amounts in solution: the neutral (HB) and the deprotonated (B^-).

3.1. Partition coefficients of nitrazepam in bile salt micelles determined by distribution methods.

The solubility of both forms of nitrazepam in bile salt solutions is practically constant till the cmc, after which increases with bile salt concentration, and finally levels off at high concentrations. In Fig. 1 plots of solubility of nitrazepam vs. surfactant concentrations are presented for sodium deoxycholate, sodium glycodeoxycholate and SDS. This increase in solubility was found to be more pronounced for the neutral form of nitrazepam and to depend on bile salt conjugation. The drug is always more soluble in glycocholate or in glycodeoxycholate, thus implying that glycine conjugation increases drug solubility, as has been found for other hydrophobic drugs [17]. The solubility of the negatively charged forms of nitrazepam does not change with the content of SDS and only the neutral forms of this benzodiazepine are increasing soluble in SDS.

The binding constant of any substance to micelles can be related with solubility through the expression $K_B^m = (S_m - S_w)/S_w C_D$ [18–21], where C_D is the micellized bile salt concentration (equal to the difference between the total surfactant concentration and the critical micellar concentration) [14,22]. Application of this equation to a neutral acid molecule (HB) and its conjugate form (B^-) yields the following expressions:

$$\frac{S_m}{S_w} = 1 + K_{HB}^m C_D \quad (2)$$

$$\frac{S_m}{S_w} = 1 + K_{B^-}^m C_D \quad (3)$$

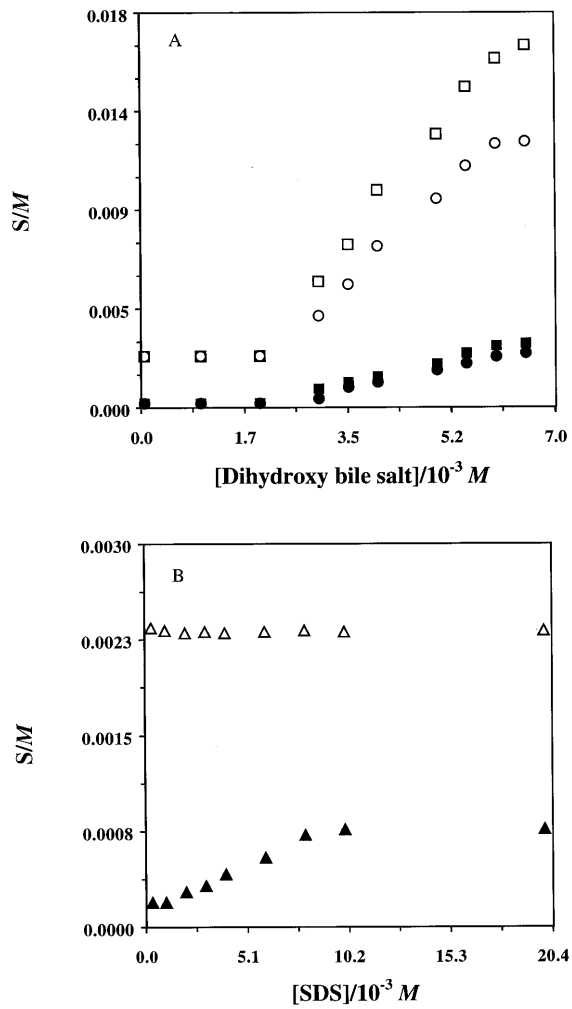

Fig. 1. Nitrazepam solubility in aqueous solutions of sodium deoxycholate (circles), sodium glycodeoxycholate (squares) and SDS (triangles) at pH 7.0 (filled symbols) and 11.5 (open symbols).

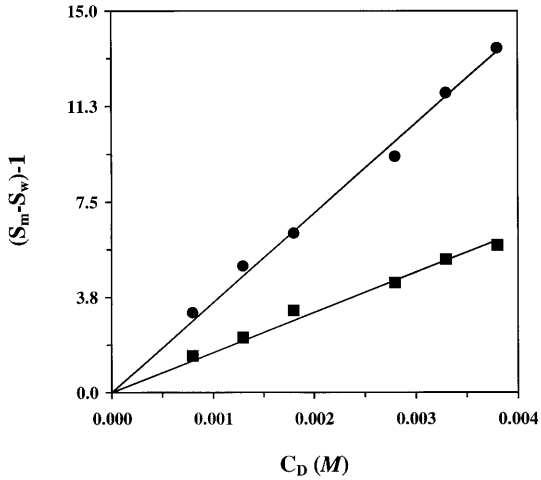

Fig. 2. Graphical representation of $(S_m - S_w) - 1$ vs. C_D of sodium glycodeoxycholate for nitrazepam at pH 7.0 (circles; $y = 3358x + 0.4886$; $R^2 = 0.992$) and 11.5 (squares; $y = 1447x + 0.3686$; $R^2 = 0.993$).

These latter equations were found to be valid only for bile salt concentrations for which the solubility changes appreciable, but not for high concentrations of surfactant where drug solubility remains constant. Plots of $(S_m - S_w) - 1$ vs. C_D are linear and from the slopes it is possible to calculate the corresponding binding constants. Such a plot for nitrazepam in sodium glycodeoxycholate is depicted in Fig. 2.

Typically, drug partition is not quantified by binding constant, but by partition coefficients. These are defined by [23,24]:

$$K_p = \frac{([B]_m/[B]_T)/C_D}{([B]_w/[B]_T)/[w]} \quad (4)$$

where [w] is the molar concentration of water and $[B]_T$ the total concentration of nitrazepam (B); $[B]_m/[B]_T$ is the mole fraction in the micelle (m); $[B]_w/[B]_T$ is the mole fraction in water (w).

The values of the binding constants can be converted into partition coefficients (K_p) through the following equations [22]:

$$K^m_{HB} = (k_{p,HB} - 1)V_\phi \quad (5)$$

$$K^m_{B^-} = (k_{p,B^-} - 1)V_\phi \quad (6)$$

where V_ϕ is the partial specific molar volume of the surfactant. Values of V_ϕ for the bile salts studied are: 0.75 $cm^3\ g^{-1}$ (sodium cholate); 0.75 $cm^3\ g^{-1}$ (sodium glycocholate); 0.77 $cm^3\ g^{-1}$ (sodium deoxycholate); and 0.77 $cm^3\ g^{-1}$ (sodium glycodeoxycholate) [25,26]. In Table 1 are presented the values of the partitions coefficients calculated from the corresponding binding constant using this approach.

3.2. Spectrophotometric determination of partition coefficients of nitrazepam in bile salt micelles

The experimental spectra on nitrazepam in micellar media are the sum of those of free and micelle bound forms of the drug. The observed absorbance, A_i, is thus the sum of the absorbance in aqueous, A_w, and in micellar solutions, A_m, and its value can be related to free and micelle bound concentrations by $A_i = \varepsilon_w [B]_w + \varepsilon_m [B]_m$. By letting $\varepsilon = \varepsilon_m - \varepsilon_w$ and $b = \varepsilon [B]_T$, and replacing these values in Eq. (4), the following equation can be obtained [24]

$$A_i = A_w + \frac{bK_pC_D}{1 + K_pC_D} \quad (7)$$

which relates experimental absorbance with partition coefficients. The values of the partition coeffi-

Table 1
Partition coefficients (M^{-1}) of nitrazepam in bile salt micelles of sodium cholate (NaC), sodium glycocholate (NaGC), sodium deoxycholate (NaDC) and sodium glycodeoxycholate (NaGDC) obtained by spectrophotometry and by solubility measurements at 25°C, $I = 0.10$ M; pH 7.0 and 11.5

Bile salt	Spectrophotometry		Solubility	
	pH 11.5[a]	pH 7.0[b]	pH 11.5[c]	pH 7.0[d]
NaC	599 ± 17	2284 ± 20	625 ± 20	2306 ± 40
NaGC	712 ± 16	2430 ± 20	739 ± 20	2440 ± 30
NaDC	1035 ± 38	3025 ± 37	1113 ± 50	3125 ± 60
NaGDC	1310 ± 40	3280 ± 42	1448 ± 50	3358 ± 40

[a] The results are the average of at least two independent experiments each with values obtained at 364, 400 and 350 nm.
[b] The results are the average of at least two independent experiments each with values obtained at 308 and 258 nm.
[c] The reported values are the average of four different measurements at 364 nm.
[d] The reported values are the average of four different measurements at 308 nm.

cients can thus be obtained by fitting Eq. (7) to the experimental absorbance data. This equation can be linearized to yield [17,24]

$$(A_w - A_i)^{-1} = (A_w - A_m)^{-1} + \frac{1}{(A_w - A_m)K_p}(C_D)^{-1} \quad (8)$$

which can be used test visually the applicability of the model and to provide approximate values of K_p to be used in the non-linear minimization.

3.3. Bile salt micelles. Partition coefficients of nitrazepam

Absorbance spectra of nitrazepam in bile salt solutions below their cmc are identical to those in the absence of bile salt. However, above the cmc, band intensity decreases as the concentration of the bile salt increases, but without changes in band maxima. This behavior was observed for both forms of the drug and for all bile salts studied.

The partition coefficients of nitrazepam between the aqueous and the micellar pseudo-phases were calculated by fitting Eq. (7) to the experimental data with correlation coefficients higher than 0.99, and the calculated values are included in Table 1. Furthermore, from the values of b it is possible to calculate the values of ε_m for nitrazepam, and the following values have been obtained (values at pH 7.0 first, and then at pH 11.5): in sodium cholate, 3390 ± 90 and 5820 ± 80; in sodium glycocholate, 3580 ± 80 and 6050 ± 70; in sodium deoxycholate 3750 ± 120 and 6370 ± 110; in sodium glycodeoxycholate, 3780 ± 110 and 6600 ± 90 (the values are the average of at least three independent measurements). In Fig. 3 the experimental values of absorbance intensity of nitrazepam are plotted as a function of C_D, for sodium deoxycholate solutions; and superimposed is the curve obtained from Eq. (7) that best describes the experimental data.

3.4. Mixed bile salt/lecithin micelles. Partition coefficients of nitrazepam

The partition coefficients were obtained only for mixed micelles of sodium cholate and sodium deoxycholate with lecithin, and for two concentrations of phospholipid, either 500×10^{-6} or 750×10^{-6} M. The values of K_p were calculated from spectrophotometric data by using the approach outlined for bile salt micelles (see above), and in Table 2 are presented the values for nitrazepam in the mixed micelles.

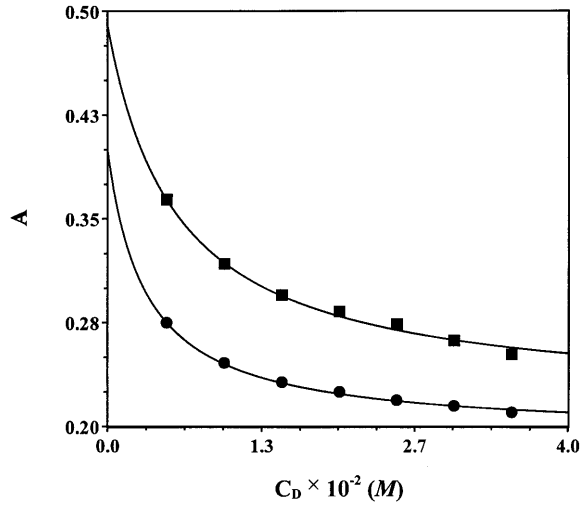

Fig. 3. Absorbance intensity (A) of nitrazepam, obtained at pH 7.0 (at 308 nm, circles) and 11.5 (at 364 nm, squares) as a function of C_D for sodium deoxycholate solutions; the line represents the best fit of Eq. (7) (see text), obtained by non-linear regression.

Table 2
Partitions coefficients (M^{-1}) of nitrazepam in mixed bile salt/lecithin micelles obtained by spectrophotometry at 25°C, $I = 0.10$ M and at two lecithin concentrations

Bile salt[a]	pH 11.5[b]	pH 7.0[c]
NaC/lecithin 500 μM	576 ± 37	1340 ± 29
NaC/lecithin 750 μM	1096 ± 32	1052 ± 31
NaDC/lecithin 500 μM	1417 ± 18	2558 ± 92
NaDC/lecithin 750 μM	2012 ± 90	1659 ± 120

[a] NaC, sodium cholate; NaDC, sodium deoxycholate.

[b] The results are the average of at least two independent experiments each with values obtained at 364, 400 and 350 nm.

[c] The results are the average of at least two independent experiments each with values obtained at 308 and 258 nm.

4. Discussion

4.1. Bile salt micelles

Analysis of data in Table 1 shows that the partition constants determined by two experimental methods and using different theoretical models are similar, which provides support for the models used.

However, some comments must be made regarding nitrazepam solubility in bile salt micelles. At pH 7.0 the drug exists mainly in the neutral form, which is one order of magnitude less soluble than the negatively charged form, predominant at pH 11.5. Upon addition of bile salts the solubility of both forms increase, but with that of the neutral form to a much larger extent: in cholate micelles the increase in solubility is 37% for the neutral form and 6% for the deprotonated form. The corresponding values for the other bile salts are: 58 and 15%, for glycocholate; 13 and 5%, for deoxycholate; and 15 and 5% for glycodeoxycholate. The increase in solubility of the neutral form correlates with the known high hydrophobic properties of neutral benzodiazepines [13,17,19], for which strong hydrophobic interactions with the lipophilic steroid nucleus of bile salts are to be expected.

It is of interest to compare the solubility (partition coefficients) of nitrazepam in simple bile salt and in SDS micelles. In the latter, the head groups are sulphate ions, whereas in bile salt micelles the head groups are negatively charged carboxylate and neutral hydroxyl groups, which impart a smaller net surface charge density to these naturally occurring micelles. Thus, only the neutral forms of nitrazepam and of other benzodiazepines are increasing soluble in SDS, due to their hydrophobic character [19]. The solubility of the negatively charged form does not increase with the content of SDS, as the electrostatic repulsion between SDS sulphate groups and the negatively charged groups of nitrazepam overcome hydrophobic interactions between their non-polar fragments. A possible explanation for the solubility increase of negatively charged nitrazepam with bile salt concentration, may be provided by noting that the negative surface charge density of bile salt micelles is much less than in SDS micelles, which can allow for some hydrophobic interactions between drug and micelle. The existence of hydroxyl groups in the surface of bile salt micelles makes electrostatic repulsions between the negatively charged groups of the drug and of the micelles less severe, and thus the hydrophobic forces are not completely annulated.

It is also clear that both forms of nitrazepam interact with any of bile salts studied, although to a much larger extent with the dihydroxy bile salts. Furthermore, conjugation of the bile salts with glycine increases the solubility of nitrazepam and consequently the values of K_p. These observations are in agreement with the behavior of other pharmaceutical compounds in bile salt solutions previously investigated [17].

4.2. Mixed bile salt/lecithin micelles

Mixed micelles, which result after addition of lecithin to bile salt micelles, are larger and have a more fluid core then simple bile salt micelles, but their net surface charge density is smaller [11,13].

Analysis of data in Table 2 shows that the neutral and deprotonated forms of the drug have different behaviors when lecithin content is increased. The partition coefficients of the neutral form decrease with increasing lecithin content, and these reductions are $\approx 60\%$ for mixed micelles with sodium cholate, and $\approx 50\%$ with sodium deoxycholate. This behavior is similar to that observed for other neutral drugs that are slightly soluble in water, for which it is to be expected that they are not extensively incorporated in the hydrophobic core of the micelles [19,20]. On the other hand, this result must be contrasted with that for highly hydrophobic compounds, e.g. cholesterol, for which the partition coefficients increase significantly with lecithin content [21,27]

More striking is the increase in the partition coefficients of the deprotonated form of nitrazepam with increasing lecithin content. Recalling the explanation advanced for the increase in solubility of the negatively charged form of nitrazepam with bile salt concentration, we propose that the increase observed with increasing

lecithin content, must also be ascribed to the decrease in net surface charge of the mixed micelles, notwithstanding the possibility of the 'polar heads' of the negatively charged form of nitrazepam interacting the positive charges in the lecithin.

As a final commentary we note that mixed micelles incorporate larger amounts of hydrophobic compounds due to their large size and the fluidity of their core [13], but that incorporation of neutral amphiphilic drugs is less extensive in bile acids/lecithin micelles. These results can be relevant to benzodiazepine bioavailability by decreasing their absorption from the gastrointestinal tract.

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