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β-Blockers and benzodiazepines location in SDS and bile salt micellar systems An ESR study

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Abstract

The work here described aimed to find out the location of the different species of two families of pharmaceutical substances, namely two β-blockers (atenolol and nadolol) and two benzodiazepines (midazolam and nitrazepam) in synthetic (sodium dodecyl sulphate, SDS) and natural (bile salts-sodium cholate and sodium deoxycholate) micellar aggregate solutions. Electronic spin resonance spectroscopy studies were carried out, at 25 °C and at an ionic strength of 0.10 M in NaCl, using 5-, 12- and 16-doxylstearic acid probes (AS). The immobilization degree of solubilized stearic acid spin probes was found to vary with the position of the nitroxide group in the sequence 5-doxylstearic acid > 12-doxylstearic acid > 16-doxylstearic acid for SDS and 12-doxylstearic acid > 5-doxylstearic acid > 16-doxylstearic acid for both bile salts investigated. Therefore, from the rotational correlational time values obtained, it can be inferred that the structure of bile salt micelles is markedly different from that of SDS micelles and the results suggest that the bile salt micelles studied have similar structure independently of differences in the molecular structure of the respective bile salts.

Drug location studies were performed at pH 4.0 (SDS solutions) or 7.0 (bile salt solutions) and 10.8 in order to study the effect of the drug ionisation on its relative position on micelles. The results have shown that drug location is controlled by the (i) drug hydrophilicity and acid/base properties, with the more soluble compound in water (atenolol) exhibiting smaller variation of rotational correlational time (in SDS and bile salts solutions), and with both β-blockers exhibiting smaller deviations in the protonated forms and (ii) the bile salt monomers, with the dihydroxylic bile salt (deoxycholate) producing larger differences.

The work described herein allow us to conclude that the (protonated) β-blockers are probably located on the surface of the detergent micelles, and linked to them by means of essentially electrostatic forces, while the (neutral) benzodiazepines are probably located deeper in the interior of the micelles.

Keywords: β-Blockers; Benzodiazepines; SDS; Bile salts; Micelles; ESR spectroscopy; Doxylstearic acid probes

1. Introduction

Bile acids participate in many different physiological processes. For example, the salts of these acids are involved in intestinal hydrolysis and also act as emulsifying and solubilizing agents for neutral fats [1–3]. The term bile acid covers several derivatives of cholic acid which differ in the number and position of α-hydroxyl substituents. The acids have a rigid and planar "cholesterol like" ring structure which is solubilized in water by glycine or taurine residues [4].

When bile salts are dissolved in water, they form micellar aggregates above a critical concentration (cmc) as do linear ionic surfactants [5–8]. Typical ionic detergents such sodium dodecyl sulphate (SDS) contain a flexible linear chain and aggregate in

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spherical or ellipsoidal micelles with the paraffin chains forming a liquid hydrocarbon core with a charged surface [9]. In contrast, bile salt micelles have neutral surface with a high amount of hydroxyl groups exposed to the aqueous phase. The micelles can be crudely depicted by broad cylinders: the hydrophobic part of the steroids form the core whereas the hydroxy and carboxylate groups project from the outside walls [10,11].

Because the molecular structure of bile salts is very different from that of conventional detergents [9,12], the bile salts exhibit unique behaviour with respect to self-association, aggregation and molecular solubilization [1]. Size and aggregation number is usually much smaller than those of micelles formed by alkyl chain detergents [13–15].

In conventional detergents, solubilization sites include the micellar surface, the palisade layer and the hydrophobic inner core [16]. Analogous binding sites are not present in the smaller, more rigid bile salt micelles [12,13,17]. Instead, solubilization of hydrophobic compounds is accomplished through favourable interactions with the hydrophobic surfaces of the bile salt micelles, primarily at the C_{18} and C_{19} sites. The resulting solubilization microenvironment in bile salt micelles are highly apolar [12,18].

Many pharmacologically active compounds are amphiphilic or hydrophobic molecules, including phenothiazines and benzodiazepine, tranquilizers, analgesics, antibiotics, tricyclic antidepressants, antihistamines, anticholinergics, β-blockers, local anesthetics [19,20]. These compounds may undergo different kinds of association, and their site of action in the organism is frequently the plasma membrane. Even if their target is intracellular, the interaction with this first barrier plays a fundamental role [19]. In this manner, the position and orientation of the drug in the bilayer is of great importance, especially when the drug can act directly on the membrane, or can interact with molecules located in it, like enzymes or receptors.

Advances in the understanding of the effect of drugs on the structural and dynamical properties of membrane components have been achieved through the use of spectroscopic (EPR, NMR, fluorescence, Fourier transform infrared) and other biophysical techniques (X-ray and neutron diffraction, differential scanning calorimetry (DSC), titration calorimetry) [21].

The use of spectroscopic probes, which are located at different depths in the membrane and whose spectral characteristics are sensitive to the surrounding environment, is particularly suited for studies with natural and model membranes, micelles or proteins [21–25]. These probes can be used to determine proximity relationships within the membrane, as well as to evaluate quantitatively the interaction of drugs with the bilayer. Generally, the molecular probes are chosen for their spectroscopic handles and the ability to interpret the observations made. Experiments utilizing molecular probes of micro and nanoscopic structures rely on the location of the probe molecule, frequently assuming the location based on a particular feature [26–28].

ESR spin labeling has assumed an important role as structural instrument providing information about the topology and dynamics of aggregation processes of natural or synthetic surfactants, at concentrations below and above the critical micellar concentrations (cmc). The information concerning micellar interior construction as well as micellar size is necessary to interpret the behaviour of drug solubilization by bile salt micelles [29].

Recent works include the application of spin label studies on the interactions of several compounds in heterogeneous systems, giving complementary structural information and allowing the sites of drug location in different environment (micellar or bilayer) to be draw. The authors have demonstrated that theses spectroscopy techniques are a valuable tool to investigate changes in the backbone conformation and dynamics. This may be of major importance, for instance, for peptides and small proteins when they bind to cell membranes [30–32].

At present, however, information on the micellar location of pharmaceutical drugs in emulsification agents used in pharmaceutical industry is not complete. Thus, we tried to, on the one hand, obtain more information on micellar interior structure of bile salts by using the spin label method, which has already been applied to examine the interior states of biological membranes, liposomes, bile salt-lecithin mixed micelles, and synthetic surfactants such as sodium dodecyl sulfate and hexadecyltrimethylammonium bromide [33,34]. On the other hand, three doxylstearic acid probes were used to study the location of the different species of two β-blockers (atenolol and nadolol) and of two benzodiazepines (midazolam and nitrazepam) in micellar solutions of two bile salts, sodium cholate and sodium deoxycholate, and one synthetic detergent, sodium dodecyl sulphate. The paramagnetic centres of 5- and 16-doxylstearic acid spin labels are, respectively, near the micelle/water interface and deep within the micelle bilayer, and incorporation of these spin probes into micelles results in distinctive EPR spectra.

2. Experimental

2.1. Reagents and solutions

All compounds were used as received: β-blockers (atenolol and nadolol) and bile salts (sodium cholate and sodium deoxycholate), from Sigma. Benzodiazepines (midazolam and nitrazepam) were kindly provided by Hoffman-La Roche, sodium dodecyl sulphate, from Aldrich, and all other chemicals were from Merck (grade *pro analysi*). Solutions were prepared with double deionized water (conductivity less than 0.1 $\mu S\ cm^{-1}$).

Spin probes 5-, 12- and 16-doxylstearic acid (doxyl = 4,4-dimethyl-3-oxazolidinyloxy) (abbreviated as 5-AS, 12-AS and 16-AS, respectively) were obtained from Sigma in the solid state and were used without further purifications. The spin probes were solubilized in chloroform and kept in the dark at 2–8 °C.

Bile acid solutions were prepared by rigorous dilution of a stock solution, whose concentration has been determined by conductimetric titration with HCl 0.1 M (Merck, *Titrisol*), added by means of a Crison 2031 piston buret and using a Crison Micro CM 2202 conductivity meter. For all solutions studied, the ionic strength was adjusted to 0.1 M with NaCl.

2.2. pH measurements

The automatic system used to measure pH has been described elsewhere [35]. Calibrations were performed by the Gran method [36] relative to hydrogen ion concentration, using strong acid/base titremetry. Aliquots of strong base or strong acid were added to 20 ml of the stock solution to adjust $-\log[H^+]$ measurements.

2.3. Sample preparation

Spin probes dissolved in chloroform were evaporated to dryness with a stream of nitrogen to yield a thin film. The resultant film was dispersed in the appropriate NaCl solution and vortexed.

Incorporation of the surfactant and/or drug in study was accomplished by incubating solutions in the dark for 6–12 h at room temperature, which were prepared by rigorous dilution of a stock solution.

For minimization of possible modification of micellar structure by the addition of spin probe, measurements were made at surfactant concentrations of 1.0×10^{-2} M for SDS, 1.0×10^{-2} M for sodium cholate and 6.0×10^{-3} M for sodium deoxycholate. The spin probe concentration was in al cases at 1.0×10^{-4} M.

The concentration of the four drugs was kept constant (5.0×10^{-6} M) and all essays were performed at pH values ≈11.0 and 4.0 (SDS solutions) or 7.0 (bile salt solutions).

It is important to refer that, since micelles increase in size with increasing surfactant concentration above the critical micellar concentration (cmc), it is assumed that concentrations 13–60 times the cmc (SDS: 1.4 mM; sodium cholate: 6.0 mM; sodium deoxycholate: 2.5 mM) [37,38], depending on the type of surfactant molecules, the effect of solubilized spin probe (at 1.0×10^{-4} M concentration) on the size of micelles is minimal [29], thus implying that the dynamic equilibrium between monomers and micelles is not perturbed. The pK_a values for bile salts are [39]: cholate 4.66 ± 0.01; deoxycholate 4.88 ± 0.01.

2.4. EPR measurements

Measurements were carried out in capillary tubes using a spectrometer Brüker EMX at 25 °C. The spectrometer settings were: modulation frequency, 100 kHz; modulation amplitude, 1.0 G; microwave power, 8 mW; scan range, 80 G; scan rate, 1 G/s; time constant, 20.48 s. The measurements were performed in triplicate.

2.5. Effective rotational correlational times τ_1

τ_1 values were determined from ESR spectra, using the expression by Cannon et al. [29,40] for fast motion ($10^{-4} < \tau_1 < 10^{-9}$ s):

$$\tau_1 = 6.5 \times 10^{-10} W_0 \left[\left(\frac{h_0}{h_{-1}} \right)^{1/2} + \left(\frac{h_0}{h_{+1}} \right)^{1/2} - 2 \right]$$

where W_0 is the peak-to-peak linewidth of the midfield line and h_0, h_{-1} and h_{+1} are the peak-to-peak heights of the mid-, high- and low-field lines, respectively.

No correction for inhomogeneous broadening from unresolved hyperfine structure and modulation broadening were applied. Effective rotational correlational times are reproducible, within experimental error, for all the measurements in micelles of SDS and both bile salts [41,42].

3. Results and discussion

Drug location using AS probes was performed at pH 4.0 (in the case of bile salt micelles at pH 7.0, to obviate the problem of precipitate formation) and pH 10.8 in order to study the effect of drug ionisation on its relative position in micellar aggregates.

3.1. τ_1 values in SDS micelles

τ_1 values obtained of the 5-AS, 12-AS and 16-AS in micellar solutions of SDS alone or in the presence of different species of atenolol, nadolol, midazolam and nitrazepam are presented in Table 1.

The analysis of the results shows that the values of rotational correlational time, τ_1, of AS probe incorporated in SDS micelles decrease in the following order: 5-AS > 12-AS > 16-AS, which indicates a different location for the nitroxide label in the SDS aggregates. Thus, the 5-AS probe is the more immobile due to the steric hindrance between the ring of the nitroxide group of this probe and both with the hydrocarbon chains and the polar areas of the SDS micelles.

The τ_1 values presented in Table 1 show that none of the drug species that were studied affects the mobility of the 16-AS probe, that is located in the core of the micelle which allow us to infer that the location of the aforementioned drugs is not closer to the micelle'core. The τ_1 values of the three probes remain also unchanged by the deprotonated species of the nitrazepam, which corroborates the existence of electrostatic repulsive forces between the B^- species of this benzodiazepine and the SDS sulphate groups. It is important to refer that the pK_a values are p$K_{a1} = 2.98 \pm 0.01$ and p$K_{a2} = 10.55 \pm 0.03$ [35,43], and thus only two forms of the drug exist in significant amounts in solution: the neutral (HB) and the deprotonated (B^-). The observations above mentioned are in agreement with the behaviour of these pharmaceutical compounds in anionic surfactant solutions observed in previous studies [28,43].

Regarding the factors that influence the effective rotational correlational times, the data show that the τ_1 values for nadolol are always slightly higher than for atenolol, and that, for both drugs the τ_1 values are related with the medium acidity. This pH dependence is related to drug deprotonation and to the consequent weaker polar/electrostatic interactions of the neutral form of β-blockers apolar chains of SDS. The pK_a values for β-blockers are [35,43]: atenolol 9.29 ± 0.05; nadolol 9.52 ± 0.02. So, at the lower pH the drugs are fully protonated, whereas at pH 10.8 they exist predominantly in the neutral form.

Table 1
Rotational correlational times (s) of the 5-, 12- and 16-doxylstearic acid probes (AS) in micellar solutions of SDS (1.0×10^{-2} M), alone or in the presence of different species of atenolol, nadolol, midazolam and nitrazepam, obtained by Electronic Spin Resonance (ESR) spectroscopy, at 25 °C and at an ionic strength of 0.10 M in NaCl; pH 4.0 and 10.8

Drug	τ_1 (s)					
	pH 4.0			pH 10.8[a]		
	5-AS	12-AS	16-AS	5-AS	12-AS	16-AS
0	1.4×10^{-9}	9.0×10^{-10}	5.3×10^{-10}	1.4×10^{-9}	9.1×10^{-10}	5.4×10^{-10}
Atenolol	1.5×10^{-9}	9.5×10^{-10}	5.3×10^{-10}	1.9×10^{-9}	9.2×10^{-10}	5.4×10^{-10}
Nadolol	1.6×10^{-9}	9.6×10^{-10}	5.3×10^{-10}	2.2×10^{-9}	9.2×10^{-10}	5.4×10^{-10}
Midazolam	2.4×10^{-9}	1.1×10^{-9}	5.3×10^{-10}	3.0×10^{-9}	9.3×10^{-10}	5.4×10^{-10}
Nitrazepam	2.9×10^{-9}	1.2×10^{-9}	5.3×10^{-10}	1.4×10^{-9}	9.1×10^{-10}	5.4×10^{-10}

The concentration of the four drugs was kept constant (5.0×10^{-6} M). τ_1 values were determined from ESR spectra, using the expression by Cannon et al. [29,40] for fast motion, as described in Section 2. The results are the average of at least three independent experiments.
[a] For nitrazepam the pH value was 11.5.

On what concerns the differences in τ_1 values between atenolol and nadolol in SDS solutions, we recall that atenolol is a more hydrophilic drug, as quantified by its higher solubility in pure water and lower in micelle solutions [35], and it is reasonable to expect that they are not fully incorporated in the hydrophobic core of SDS micelles.

From analysis of Table 1, we also point out that SDS solutions in presence of midazolam shows higher τ_1 values for the 5-AS probe and that the increase is more pronounced at pH 10.8, for which hydrophobic interactions with the lipophilic side chains of SDS are to be expected. The pK_a values for midazolam [35,43] is 5.91 ± 0.01. Furthermore, it is also clear that the neutral forms of midazolam and nitrazepam exhibit practically identical τ_1 values, which support the known high hydrophobic properties of deprotonated benzodiazepines.

The most striking result is related to a higher variation of the τ_1 values that is verified for the 12-AS probe in micellar solutions of SDS in the presence of the protonated forms (pH 4.0) of atenolol, nadolol and midazolam, in comparison with the values calculated in presence of the neutral forms of those same drugs. On the contrary, the neutral forms of these two β-blockers and of the benzodiazepine affect mainly the τ_1 values of the 5-AS probe. A possible explanation for the results obtained may be provided by emphasizing that for both β-blockers in anionic micellar media, SDS counterions exchange with other cationic species in solution, an assumption of the PIE model [35,43]. In this model, the micelle surface is assumed to act as an ion-exchanger, e.g. the distribution of proton and alkaline metal ions in solution of anionic micelles is described by an ion-exchange constant. Within the framework of the PIE model, the equilibrium in anionic micellar media was described by the set of equilibria that characterized their dissociation and the partition (K^m) of protonated and neutral species between micelles and the solution.

Contrarily, for both benzodiazepines no ionic exchange of Na^+ ions with other protonated species was considered (Berezin model) [35,43]. These mathematical models were applied to quantify the effect of SDS micelles on the pK_a of the drugs used and the binding constants determined for neutral species were always smaller than those of positively charged (protonated) species and that the binding of negatively charged nitrazepam was 1–2 orders of magnitude smaller than binding of neutral species. The neutral forms of β-blockers bind more weakly than the corresponding forms of benzodiazepines. These observations are correlated with the results obtained in this work, once the τ_1 values of the 5-AS probe in the presence of protonated β-blockers are smaller than for 12-AS probe. In this manner, the Na^+ ions bound to the surfactant surface may exchange with other species in solution and the spin probe mobility can, in this manner, be higher. On other hand, formation of "rigid domains" can occur, which may stabilize the charged species, allowing that protonated forms of atenolol and nadolol penetrate more deeply and disturb the effective rotational correlational times of 12-AS probe.

Contrastingly, at pH 10.8 the β-blockers exist mainly in the neutral form, thus inferring that the species increase more significantly the τ_1 values of 5-AS probe.

The behaviour observed in this work is in accordance with what was described by Kawamura et al. [29], and the τ_1 values for 12-AS and 16-AS probes that were obtained are of the same order of magnitude as the ones published by those authors. For 5-AS probe, τ_1 values are always lower, probably due to the electrolyte concentration used. The effect of counterion concentration in the micellar aggregation of synthetic and natural surfactants has been extensively studied by different authors [44–47] and is a general statement that the addition of an inert electrolyte, which may alter the ionic strength of the solution, will promote an increase of the micellar aggregates of the surfactants [5,6,13,20,45,46] and, therefore, significant ionic variations in the surfactant surface can occur. According to Carey et al., the effects of counterion on the micellar stabilization are mainly mediated by progressive neutralization of the ionic charges of anionic detergents [6,13,37,45,48], thus implying that the Na^+ ions can affect the spin probe mobility at the surface. A solicitous analysis of the Kawamura work shows the omission of experimental conditions, namely the ionic strength value. This parameter influences the τ_1 values and should always be specified in order to establish valid comparisons between the results.

Table 2
Rotational correlational times (s) of the 5-, 12- and 16-doxylstearic acid probes (AS) in micellar solutions of sodium cholate (1.0×10^{-2} M), alone or in the presence of different species of atenolol, nadolol, midazolam and nitrazepam, obtained by Electronic Spin Resonance (ESR) spectroscopy, at 25 °C and at an ionic strength of 0.10 M in NaCl; pH 7.0 and 10.8

Drug	τ_1 (s)					
	pH 7.0			pH 10.8[a]		
	5-AS	12-AS	16-AS	5-AS	12-AS	16-AS
0	1.2×10^{-9}	1.8×10^{-9}	7.3×10^{-10}	1.3×10^{-9}	1.5×10^{-9}	7.6×10^{-10}
Atenolol	1.3×10^{-9}	1.9×10^{-9}	5.5×10^{-10}	1.3×10^{-9}	2.4×10^{-9}	3.6×10^{-10}
Nadolol	1.3×10^{-9}	2.0×10^{-9}	6.7×10^{-10}	1.4×10^{-9}	3.2×10^{-9}	7.4×10^{-10}
Nitrazepam	1.7×10^{-9}	3.7×10^{-9}	7.3×10^{-10}	1.3×10^{-9}	1.5×10^{-9}	8.0×10^{-10}

The concentration of the four drugs was kept constant (5.0×10^{-6} M). τ_1 values were determined from ESR spectra, using the expression by Cannon et al. [29,40] for fast motion, as described in Section 2. The results are the average of at least three independent experiments.
[a] For nitrazepam the pH value was 11.5.

The τ_1 values of the 5-AS and 12-AS probes in presence of the two β-blockers are clearly lower than the values obtained in presence of the two benzodiazepines. This way, the results obtained allow us to say that there is a difference in the location and extent of the association of the two kinds of drugs in the micelle. In this way, neutral forms of β-blockers, are predominantly incorporated in micellar surface of SDS, while neutral benzodiazepines, due to their hydrophobic character, are located in the interior of SDS micelles.

3.2. τ_1 values in bile salt micelles

The τ_1 values of the 5-AS, 12-AS and 16-AS probes, in micellar solutions of sodium cholate and sodium deoxycholate are presented in Tables 2 and 3, respectively.

The results obtained show that the immobilization level of the stearic acid spin probes varies for both the bile salts studied, according to the position of the nitroxide group, in the following order: 12-AS > 5-AS > 16-AS, showing of that the τ_1 values of 16-AS for the above-named bile salt solutions is rather close to τ_1 of 5-AS. The behaviour observed is qualitatively similar for both sodium cholate and sodium deoxycholate.

The variations observed for sodic salts of the cholic and the deoxycholic acids have the same order of magnitude as those previously observed with sodium taurodeoxycholate, taurochenodeoxycholate and sodium tauroursodeoxycholate, but are slightly lower than the results published by others works [29]. This difference as was previously mentioned is probably due to different sodium concentration. As a matter of fact, the presence of electrolyte not only changes the physical properties of the micelles but also changes the interaction pattern of micelles with a molecule in the bulk solvent, which means that addition of counterions in a micellar medium will affect not only micellar parameters but also the ionic environment near the surface of the micelles [13,47,49,50].

As can be observed from Tables 1 and 2, the τ_1 values of 12-AS and 16-AS for bile salt solutions are significantly larger compared with those for SDS. Therefore, it can be inferred that the micellar structure of bile salts is markedly different from SDS. This different behaviour of 12-AS for both bile salt solutions is observed even for biomembranes and model membranes [29]. 12-AS incorporated by erythrocyte membrane reflects the lipid-protein interaction more strongly than the other probes. Thus, we deem that 12-AS is most appropriate for searching the interior of bile salt micelles and reveal the suitable localization of the studied drugs.

Kawamura et al. [29] proposed that a stearic acid spin probe can be considered to penetrate through the bile salt micelle by taking account of the fact that the molecular length of spin probe is longer than that of bile salts. Based on this, the nitroxide group of the 12-AS probe is to be found in the hydrophobic moiety of the bile salts molecules and is the most strongly immobi-

Table 3
Rotational correlational times (s) of the 5-, 12- and 16-doxylstearic acid probes (AS) in micellar solutions of sodium deoxycholate (6.0×10^{-3} M), alone or in the presence of different species of atenolol, nadolol, midazolam and nitrazepam, obtained by Electronic Spin Resonance (ESR) spectroscopy, at 25 °C and at an ionic strength of 0.10 M in NaCl; pH 7.0 and 10.8

Drug	τ_1 (s)					
	pH 7.0			pH 10.8[a]		
	5-AS	12-AS	16-AS	5-AS	12-AS	16-AS
0	1.5×10^{-9}	2.1×10^{-9}	7.6×10^{-10}	1.5×10^{-9}	2.0×10^{-9}	7.7×10^{-10}
Atenolol	1.6×10^{-9}	2.4×10^{-9}	6.2×10^{-10}	1.8×10^{-9}	3.1×10^{-9}	5.8×10^{-10}
Nadolol	1.8×10^{-9}	2.4×10^{-9}	7.3×10^{-10}	2.0×10^{-9}	3.8×10^{-9}	7.5×10^{-10}
Nitrazepam	2.0×10^{-9}	4.1×10^{-9}	7.6×10^{-10}	1.5×10^{-9}	2.0×10^{-9}	8.2×10^{-10}

The concentration of the four drugs was kept constant (5.0×10^{-6} M). τ_1 values were determined from ESR spectra, using the expression by Cannon et al. [29,40] for fast motion, as described in Section 2. The results are the average of at least three independent experiments.
[a] For nitrazepam the pH value was 11.5.

lized. On the other hand, the nitroxide ring of the 5-AS probe is located near the polar area of these surfactants and is to be found partially in contact with the apolar portion of the above-mentioned molecules. The nitroxide ring of the 16-AS probe is projected towards the exterior of the micelles and, consequently, its τ_1 value is smaller. The 5-AS probe may be, therefore, more strongly immobilized than 16-AS.

Trihydroxy bile salts, like sodium cholate, have a smaller aggregation number compared with dihydroxy bile salts or with conventional aliphatic surfactants [6,12,13,17,51]. For the sodium cholate micelle, a lower value of 4 and a higher value of 16 [12], have been published depending upon the method employed. Moreover, the hydrodynamic radius has been reported to be 1.4 nm for sodium cholate micelles and ≈2.0 nm for sodium deoxycholate [12]. This indicates that cholate bile salt micelles are smaller in size compared with, for instance, sodium deoxycholate.

Therefore, protonated forms of several drugs that can interact with the polar part of trihydroxy bile salt micelles may be their solubilization reduced, as has been found for some hydrophilic compounds [51]. Consequently, the number of molecules of different species of atenolol, nadolol, midazolam and nitrazepam is lesser and the τ_1 values of the 5-AS probe in micellar solution of sodium cholate is not significantly perturbed, as can be seen from Table 2.

From analysis of Tables 2 and 3 it is also clear that both β-blockers and nitrazepam exhibit the same behaviour with any of bile salts studied, although with sharper variations for the dihydroxy bile salt. Therefore, our results show that drug location depends on the number of hydroxyl groups of bile acid. This difference reflects differences in micellar structure between trihydroxy bile salts and dihydroxy bile salts, despite their steroid nucleus.

Unlike the behaviour of the 16-AS probe in SDS solutions and in presence of the deprotonated forms of the nitrazepam, the τ_1 values of 16-AS probe is affected by the already mentioned free species in solutions of both bile salts. Recalling the explanation already advanced, we propose that the negative surface charge density of bile salt micelles is lower than in SDS micelles which can allow for some hydrophobic interactions between drug and micelle. Indeed, 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 hydrophobic forces may have a strong contribution to the overall interaction. The mobility of the remaining probes is not affected by the B^- species of benzodiazepine in cholate and deoxycholate aggregates.

At pH 7.0 nitrazepam exists mainly in the neutral form, which presents a partition coefficient between the aqueous and the bile salts micellar pseudo-phases (cholate: $K_p = 2306 \pm 40\,M^{-1}$; deoxycholate: $K_p = 3125 \pm 60\,M^{-1}$) three to four orders of magnitude higher than the negatively charged form, predominant at pH 11.5 (cholate: $K_p = 625 \pm 20\,M^{-1}$; deoxycholate: $K_p = 1113 \pm 50\,M^{-1}$) [51]. So, the increase in τ_1 values of the 12-AS probe, and more feebly, of the 5-AS is related with the high hydrophobic properties of neutral benzodiazepines for which strong hydrophobic interactions with the apolar steroid nucleus of bile salts are to be expected. The variations observed of the τ_1 values in the presence of the neutral species of nitrazepam is always clearly higher in solutions of sodium deoxycholate in comparison with the values obtained in sodium cholate solutions, as already mentioned.

In what concerns the factors that influence the values of τ_1 in the presence of β-blockers, the data show (Tables 1 and 2) that for the same bile salt, the τ_1 values in nadolol solution are always higher than for atenolol solution, and that the neutral forms of the drugs greatly affect the τ_1 values of the 12-AS probe in micellar solutions of sodium cholate and sodium deoxycholate. Therefore, they are probably incorporated nearer to the steroid core. The first observation is a direct consequence of the higher hydrophobicity of nadolol, as can be gathered from its molar solubility in water, which is nine times smaller than atenolol [52]. The latter observation is related to the predominant β-blocker form in bile salt solution. At pH 7.0 both drugs exist predominantly (>90%) in the protonated form and thus the interaction with the deprotonated carboxylate of the side chains of the bile salt micelles will be mainly electrostatic (opposite charges). At the higher pH (10.8) the drugs are present mainly in the neutral form, thus, precluding charge attraction by the carboxylate side chains of the micelles. To account for the behaviour of atenolol and nadolol we note that these drugs are amphiphilic [53] and appreciably soluble in water and thus, it is reasonable to expect that they are not fully incorporated in the hydrophobic core of simple bile salt micelles, in contrast with what is found for cholesterol [54,55]. The remark that both forms of β-blockers affect mainly the τ_1 values of the 12-AS probe in micellar solutions of sodium cholate and sodium deoxycholate, suggest that, notwithstanding the recognition that drug-bile salt micelle have a large polar/electrostatic component, the lipophilic part of the drugs are incorporated in the bile salt micelle interior. Our results are related and corroborate the large partition coefficients observed at pH 7.0 and obtained in previous works [52]. The K_p values for the protonated β-blockers are [52]: atenolol/cholate, $793 \pm 32\,M^{-1}$; atenolol/deoxycholate, $1074 \pm 43\,M^{-1}$; nadolol/cholate, $1597 \pm 40\,M^{-1}$; nadolol/deoxycholate, $2130 \pm 90\,M^{-1}$. For the neutral species the K_p values are: atenolol/cholate, $192 \pm 10\,M^{-1}$; atenolol/deoxycholate, $244 \pm 11\,M^{-1}$; nadolol/cholate, $319 \pm 10\,M^{-1}$; nadolol/deoxycholate, $526 \pm 25\,M^{-1}$. Once again, the data show that the pH dependence is related to drug deprotonation and the consequent weaker polar/electrostatic interactions of the neutral drugs. The dependence with the number of hydroxyl groups can be explained by invoking the accepted model for simple bile salt micelles in which dihydroxy bile salts form secondary micelles and can incorporate more deeply hydrophobic compounds due to their large size and rigid nucleus [6,12,13,17].

From Tables 2 and 3 it also clear that, with the exception of the neutral forms of the nitrazepam, all the other species of the drugs in study, mainly the neutral form of atenolol, when present in solution of both bile salts, increase the mobility of the 16-AS probe. A possible explanation for the effective rotational correlational times decrease may be provided by noting that the neutral species of atenolol owns the

peculiarity of disturb and modify more largely the micellar structure.

A model of a spherical micelle such as that for a SDS micelle is not applicable to bile salt micelles, and these aggregates are better described as disklike micellar model: as the concentration approaches the cmc, bile salt molecules form dimers or slightly larger aggregates [54]. These primary micelles are mainly stabilized by hydrophobic interactions wherein the apolar surfaces of adjacent molecules (lipophilic steroid nucleus), which are partially sequestered from the solvent, whereas the polar groups tend to remain accessible to, and solvated by, the aqueous solution. Upon a further increase in concentration, the primary micelles of dihydroxy bile salts, like deoxycholate, tend to combine into larger aggregates, mainly stabilized by intermolecular hydrogen bonding between some of the hydroxyl groups. Position and orientation of the hydroxyl groups play an important role in the aggregation process. In the primary aggregation step, hydrophilic "islands", on a generally hydrophobic surface, reduce the stabilization provided by the hydrophobic interaction [56]. In contrast, the secondary aggregation is essentially due to the hydrogen bond stabilization provided by properly oriented hydroxyl groups [57,58].

Dihydroxy bile salts form micelles of larger aggregation number and more rigid structure than that of trihydroxy surfactants [29,40,59–63], which could explain the fact that the higher τ_1 values presented in this work happen precisely with the earliest.

The fact that the counterion dissociation of bile salt micelles is much larger than that of SDS micelle [6,12,52], can also be explained by the same model. The polar head-group charged density on a micellar surface is lower (in SDS the headgroups or sulphate ions, whereas in bile salt micelles the head-groups are charged carboxylate and neutral hydroxyl groups), and, consequently, the degree of sodium binding is smaller for the sodium cholate micelles than in the case of SDS micelles.

4. Conclusions

A reliable and convenient method to assess the solubilization site of a solubilizate in an aqueous micellar system is provided by ESR study [34,64,65]. The results obtained allow us to verify the existence of different types of interaction between drug and micelle. These interactions that determines the location of the solute in the micelles vary according to the nature of the solubilized species and of the surfactant.

Since the protonated species of midazolam and nitrazepam are more strongly associated with the SDS aggregates than with the corresponding forms of the two β-blockers, it can be assumed that, in consequence of the high lipofilicity of the benzodiazepines, these will penetrate more extensively the interior of the micelles than atenolol and nadolol, being, therefore, less predisposed to ionic exchange.

Nitrazepam, the drug with the highest association constants and partition coefficients in any of the heterogeneous systems studied in this work [51], is likely to establish stronger and more extensive interactions with the bile salts' simple micelles. On the other hand, the neutral form of this benzodiazepine is more incorporated in the interior of the bile salt micelles, as can be concluded from the analysis of the results obtained in the ESR studies.

The identification of the location of the drugs studied in the micelles of SDS and bile salts allowed us to confirm the existence of several types of interaction and distribution of the two types of family of drugs in the micelles that were formed. Since the micelle is a dynamic entity, it is understandable that a clear distinction bewteen the probable solubilization areas of the protonated and neutral forms of the two β-blockers and of the two benzodiazepines is not observable. However, the following locations may be predominant: (I) adsorption of the β-blockers ionized species on the surface of the micelle, i.e., on the water/micelle interface; (II) between the hydrophilic groups (neutral species of the β-blockers); (III) near the micellar surface, radially oriented in the micelle (protonated forms of the benzodiazepines); (IV) interior of the micelle (neutral forms of the benzodiazepines).

As a final commentary it can be said that the information on the core structure of the micelles as well as the micellar size must be considered to explain the differences in the effective rotational correlational times of stearic spin probes in various surfactants solutions. These changes reflect modifications in the microenvironment of the molecule undergoing solubilization in the heterogeneous systems and can provide a detail picture for partitioning and location of the drugs in micelles.

The results obtained in this work can be relevant to β-blockers and benzodiazepines bioavailability by changing their absorption from the gastrointestinal tract and, mainly, in the selection of a surfactant in the manufacture of pharmaceutical compositions.

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