ESR Spectroscopy Insight on the Anticancer Drug Mitoxantrone Location in Membrane Mimetic Systems

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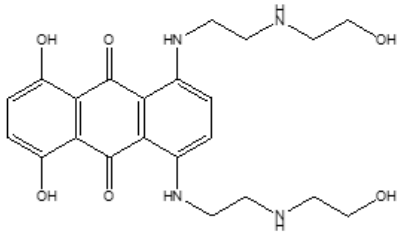
The location of mitoxantrone molecule in micelles formed by bile salts (sodium taurocholate (NaTC) and sodium taurodeoxycholate (NaTDC)) and sodium dodecyl sulfate (SDS) have been investigated by electron spin resonance (ESR) spectroscopy, using three doxylstearic acid probes (5-, 12- and 16-doxylstearic acid abbreviated as 5-DSA, 12-DSA and 16-DSA). The analysis of ESR parameters of these spin probes evidenced slower dynamics induced by mitoxantrone that vary in the following order: 12-DSA > 5-DSA > 16-DSA for both bile salts micelles and 5-DSA > 12-DSA > 16-DSA for SDS micelles. The ESR parameters are slightly sensitive to variation of pH. These results indicate that the spin probes target different regions in these aggregates.

Keywords: mitoxantrone, bile salts micelles, SDS micelles, ESR spectroscopy

Mitoxantrone is a synthetic anthracenedione-based anticancer drug developed as a doxorubicin analogue with decreased cardiotoxicity [1]. Mitoxantrone has a broad spectrum of antimor activity, being used in the treatment of advanced breast and prostate cancer, leukemia, lymphoma and multiple sclerosis [2-5]. The advantage of the mitoxantrone use relies in a reduced cardiotoxicity of anthracyclines and retaining or improving their antitumor activity. Even so, the cardiotoxicity associated with mitoxantrone treatment has been significantly reported and this cardiotoxicity depends on age and cumulative dose [6]. Its clinical usefulness is also limited by the occurrence of multidrug resistance (MDR) associated with the overexpression of ATP-binding casette (ABC) membrane transporters [7, 8].

The structure of mitoxantrone (Fig. 1) consists of a tricyclic planar chromophore substituted with two flexible side chains. Mechanism of antitumor activity of mitoxantrone is not completely understood but different studies have shown that nuclear DNA is the major target for the drug and the planar anthraquinone ring intercalates between DNA base pairs and the nitrogen-containing side chains bind the negatively charged phosphate groups of DNA [9-13]. The binding of mitoxantrone to DNA leads to the inhibition of both DNA replication and RNA transcription processes [14]. Also, mitoxantrone is a potent inhibitor of topoisomerase II, an enzyme known to be important for the repair of damaged DNA [15, 16].



mitoxantrone

Fig. 1 Chemical structure of mitoxantrone

Before reaching the nuclear DNA inside of the cell, mitoxantrone cross and interacts with cellular and nuclear membranes. Surfactant micelles with their hydrophilic surface and hydrophobic interior serve as simple membrane mimetic system that allows a controlled study of the effect of different membrane parameters on the structural dynamism of ligand molecules [17]. The amphiphilic nature of micelles allows studying the affinity of different drug molecules to the biological membranes and hence the physicochemical interactions of drugs with surfactant micelles can be visualised as an approximation for drug–membrane interactions [18-22].

The bile salts have a common chemical structure quite different from synthetic surfactants. They have a core comprising a large, rigid and hydrophobic steroid moiety with attached hydrophilic groups (typically two or three hydroxyl groups) and an anionic carboxyl head group. This specific structure results in an aggregation pattern and a micellar structure much different from those of conventional surfactants, such as sodium dodecyl sulphate [23, 24].

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Electron spin resonance (ESR) spectroscopy is a well-known method to study drug interactions with natural and model membranes (micelles, liposomes) with the use of spin probes which are located at different depths in the membrane and whose spectral characteristics are sensitive at the surrounding environment [25-27]. The spin probe method has been also used to study the interaction with commercially available proteins like BSA and HSA [28-31] or proteins from biological fluids such human blood or tears [32-34]. ESR spin labelling method that consist in covalently attachment of a paramagnetic moiety to a molecule has been used to obtain information about the topology and dynamics of aggregation processes of natural and synthetic surfactants, the interaction of different compounds in heterogeneous systems and the drug location sites in different micelles [35-38].

Previously we examined the equilibrium interactions of mitoxantrone with micelles made from different synthetic (sodium dodecyl sulfate (SDS), cetyltrimetylammonium bromide (CTAB), Triton X-100, Brij-35, Tween-20, Tween-80) [39-42] and natural (sodium taurocholate (NaTC) and sodium taurodeoxycholate (NaTDC) bile salts) surfactants [36] in order to assess the strength of the interaction and the importance of electrostatic and hydrophobic contribution to the drug binding.

In the present study we have extended the previous work by investigating the location of mitoxantrone molecule in micelles of two bile salts, sodium taurocholate (NaTC) and sodium taurodeoxycholate (NaTDC), and one synthetic surfactant, sodium dodecyl sulfate (SDS) using ESR spectroscopy. Also, the micellar aggregates of NaTC, NaTDC and SDS in phosphate buffer (*p*H 7.4) and carbonate buffer (*p*H 10) are examined. Three doxylstearic acid probes (5-, 12- and 16-doxylstearic acid abbreviated as 5-DSA, 12-DSA and 16-DSA) were used to provide information on the self-assembly of NaTDC, NaTC and SDS in the absence and the presence of mitoxantrone. The experiments were performed at *p*H 7.4, when mitoxantrone has two positive charges due to the protonation of amino groups on lateral chains and *p*H 10, when mitoxantrone is uncharged.

Experimental part

Mitoxantrone dihydrochloride, SDS, NaTC, NaTDC and spin probes 5-, 12- and 16-doxylstearic acid (DOXYL = 4,4-dimethyl-3-oxazolidinyloxy) (5-DSA, 12-DSA, 16-DSA) were analytical grade and supplied by Sigma Aldrich. All the compounds were used without further purification. Solutions were prepared with deionized water (18.2 MΩcm, Mili-Q water purification system). In order to minimize the possible modification of the structure of micelles by the addition of spin probes, measurements were made at surfactant concentrations of 1.00×10^{-2} M for SDS and 2.00×10^{-2} M for NaTDC and NaTC. Micellar solutions of SDS, NaTDC and NaTC were prepared in 0.1 M phosphate buffer, *p*H 7.4 and 0.1 M carbonate buffer, *p*H 10. The critical micellar concentration (CMC) of SDS in water is 9.00×10^{-3} M and this value remain practically constant for pH between 6 and 10 [43], and 1.99×10^{-3} M in 50 mM phosphate buffer *p*H 7 [44]. The CMC values for bile salts are: 3 mM for NaTDC and 4 mM for NaTC in water, and 2 mM for NaTDC and 5 mM for NaTC in 0.1 M salt solution [45].

Stock solutions of spin probes (1.00×10^{-2} M) were prepared in ethanol. To prepare samples for ESR measurements, in each case, an appropriate volume of ethanol solution of the spin probe was evaporated from a vial under a stream of inert gas. Subsequently a buffer solution (containing SDS, NaTC, NaTDC and mitoxantrone) was added to reach a spin probe concentration of approximately 1.00×10^{-4} M. To record ESR spectrum, each solution was transferred to glass capillaries and sealed.

The ESR spectra were recorded on a JEOL FA 100 spectrometer at room temperature, with the following parameters: frequency modulation of 100 kHz, microwave power of 0.998 mW, sweep time of 480 s, modulation amplitude of 1 G, time constant of 0.3 s, and magnetic field scan range of 100 G. All the measurements were made at 20 ^{0}C.

Results and discussions

In the present study three amphiphilic spin probes from the family of doxyl type spin probes (5-DSA, 12-DSA, 16-DSA) were used to investigate the location of mitoxantrone in micellar solutions of SDS, NaTDC and NaTC. The paramagnetic centers of 5-DSA and 16-DSA are near the micelle/water interface and deep within the micelle bilayer, respectively, and incorporation of these spin probes into micelles results in distinctive ESR spectra. On the other side 12-DSA senses a less hydrophobic environment around the paramagnetic center [27]. The experiments were performed at *p*H 7.4 and *p*H 10 in order to investigate the effect of mitoxantrone charge on its relative position in micellar aggregates.

The rotational correlational times of the spin probe showing an isotropic dynamic regime were determined using equation (1):

$$\tau_c = 6.51\times10^{-10}\Delta H_0\left[\left(\frac{h_0}{h_{-1}}\right)^{1/2}+\left(\frac{h_0}{h_{+1}}\right)^{1/2}-2\right] \quad (1)$$

where ΔH_0 is the peak-to-peak width (in Gauss) of the central line, and h_{-1}, h_0 and h_{+1} are the heights of the low, central and high field lines, respectively [35].

The spin probes in micellar solutions exhibit a slower dynamic compared with buffer solutions, as the ESR lines are broader and, in some cases, a two-component feature is observed for their ESR spectra. In these cases, the ESR spectral simulations were performed using the program developed by Budil et al., based on non-linear least–squares NLSL fits [46].

The ESR spectra of 5-DSA, 12-DSA and 16-DSA in SDS, NaTDC and NaTC systems in the absence and the presence of mitoxantrone are presented in Fig. 2 for *p*H 7.4 and Figure 3 for *p*H 10.

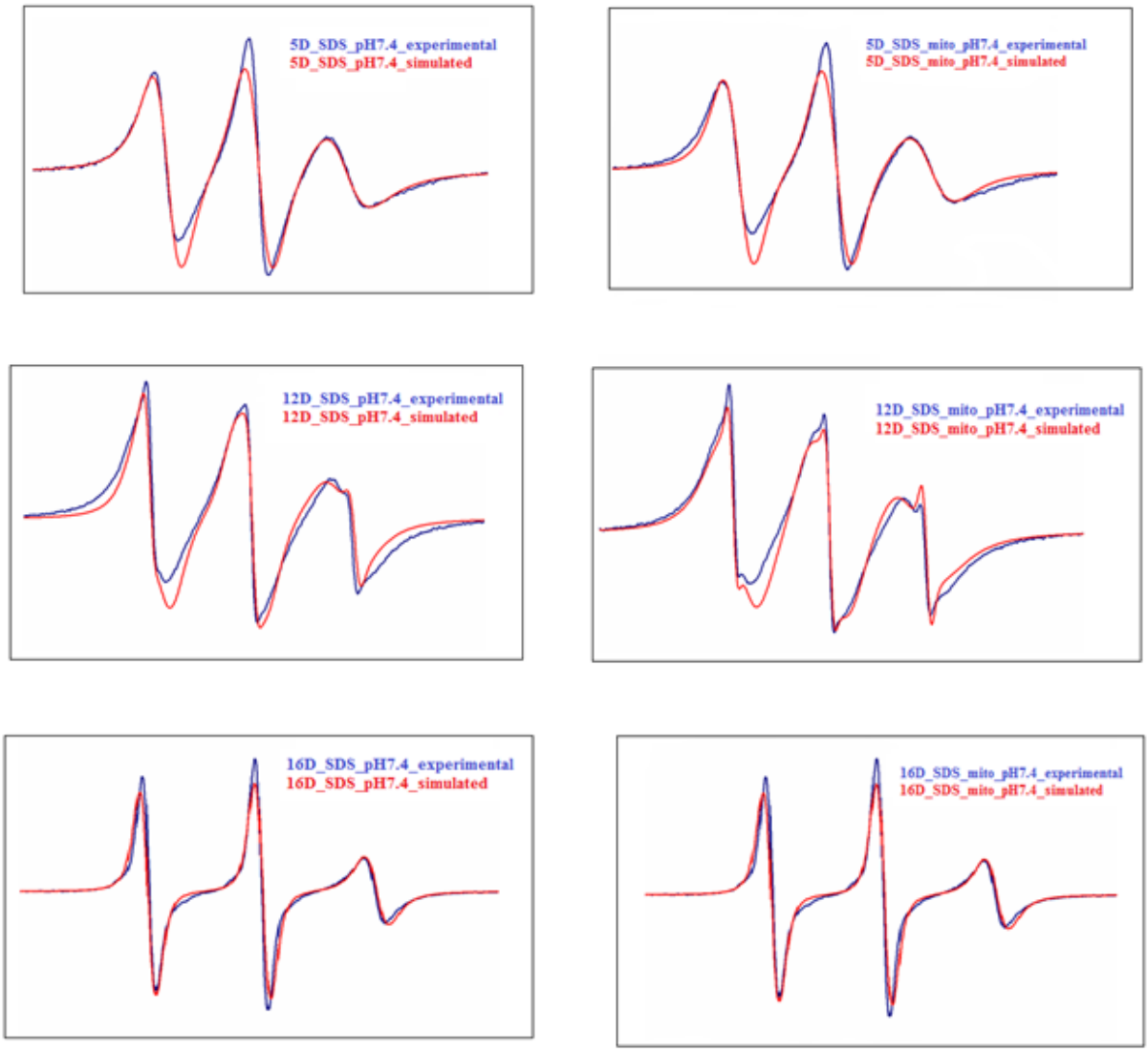

Fig. 2. Experimental and simulated ESR spectra of 5-DSA (top row); 12-DSA (middle row) and 16-DSA (bottom row) in SDS micelles in the absence and the presence of mitoxantrone (mito) in phosphate buffer *p*H 7.4

In phosphate buffer solution (*p*H 7.4) all three spin probes have the same nitrogen coupling constant (a_N) value (15.85 G) corresponding to a hydrophilic environment. At the same time, all spin probes exhibit an isotropic motion [46]. The values a_N = 15.87 G are similar in carbonate buffer, at *p*H 10 for all spin probes.

Rotational correlational time values obtained for the 5-DSA, 12-DSA and 16-DSA in micellar solutions of SDS, NaTDC and NaTC alone and in the presence of mitoxantrone are included in Table 1 for *p*H 7.4 and Table 2 for pH 10.

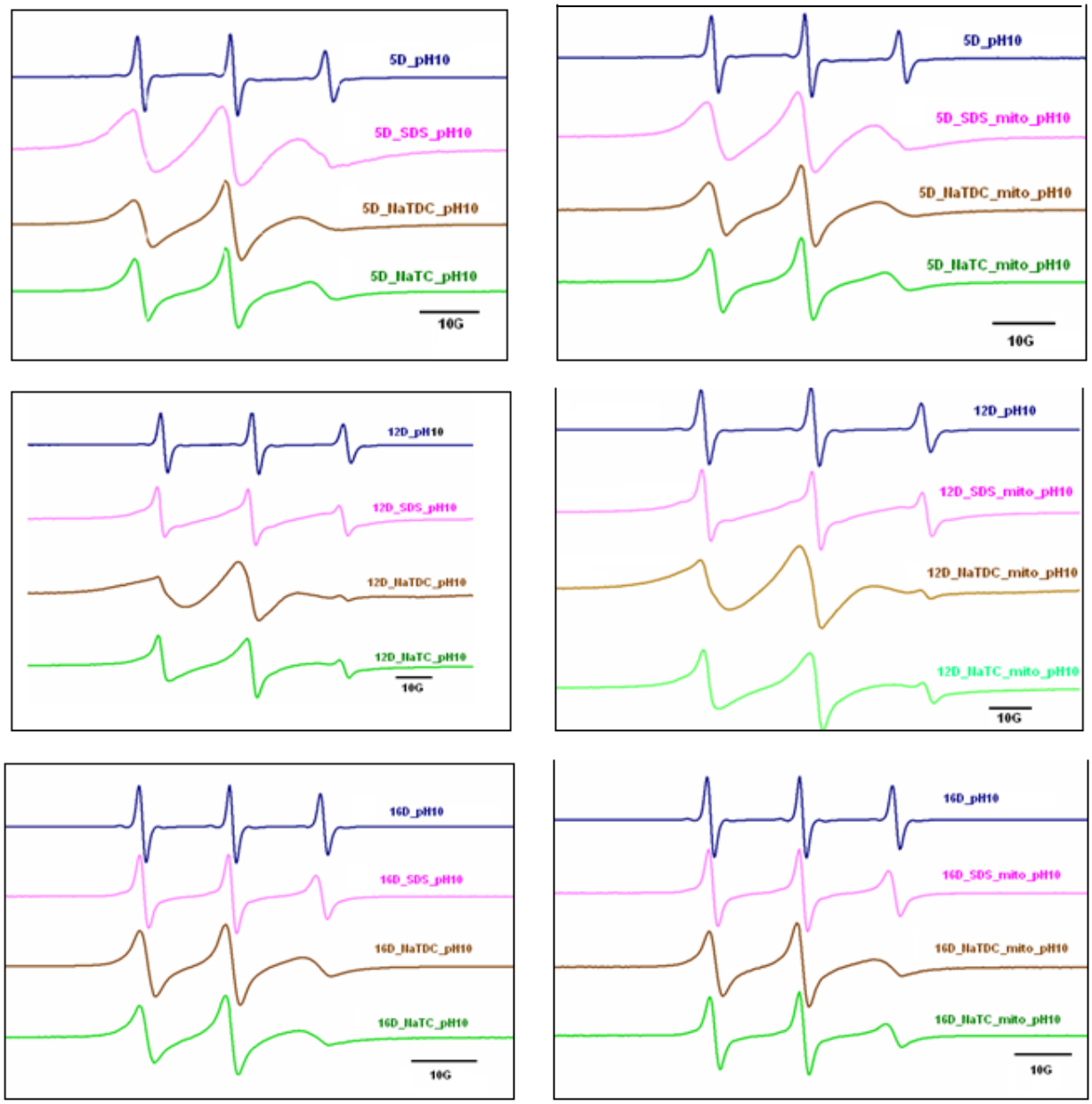

Fig. 3. ESR spectra of 5-DSA (top line); 12-DSA (middle line) and 16-DSA (bottom line) in carbonate buffer pH 10, SDS, NaTDC and NaTC micelles, in the absence and the presence of mitoxantrone (mito)

Table 1

ROTATIONAL CORRELATIONAL TIME, τ (s) OF THE 5-DSA, 12-DSA AND 16-DSA IN MICELLAR SOLUTIONS OF NaTDC (2.00×10^{-2} M), NATC (2.00×10^{-2} M) AND SDS (1.00×10^{-2} M), IN THE ABSENCE AND IN THE PRESENCE OF MITOXANTRONE (2.55×10^{-5} M) IN PHOSPHATE BUFFER, *p*H 7.4

System	5-DSA	12-DSA	16-DSA
	τ (s)	τ (s)	τ (s)
phosphate buffer, pH 7.4 [36]	2.55×10^{-10}	2.50×10^{-10}	1.23×10^{-10}
NaTDC [36]	2.96×10^{-9}	6.39×10^{-9}	2.02×10^{-9}
mitoxantrone + NaTDC [36]	3.50×10^{-9}	6.72×10^{-9}	2.44×10^{-9}
NaTC [36]	2.13×10^{-9}	3.26×10^{-9}	1.18×10^{-9}
mitoxantrone + NaTC [36]	2.56×10^{-9}	3.44×10^{-9}	1.28×10^{-9}
SDS	2.18×10^{-9}	1.81×10^{-9}	6.52×10^{-10}
mitoxantrone + SDS	2.36×10^{-9}	1.87×10^{-9}	5.31×10^{-10}

Table 2
ROTATIONAL CORRELATIONAL TIME, τ (s) OF THE 5-DSA, 12-DSA AND 16-DSA IN MICELLAR SOLUTIONS OF NATDC (2.00×10^{-2} M), NaTC (2.00×10^{-2} M) AND SDS (1.00×10^{-2} M), IN THE ABSENCE AND IN THE PRESENCE OF MITOXANTRONE (2.55×10^{-5} M) IN CARBONATE BUFFER, *p*H 10

System	5-DSA	12-DSA	16-DSA
	τ (s)	τ (s)	τ (s)
carbonate buffer, pH 10	$2.33\ x10^{-10}$	$2.25x10^{-10}$	$1.09\ x10^{-10}$
NaTDC	$3.38\ x10^{-9}$	$6.19\ x10^{-9}$	$1.77\ x10^{-9}$
mitoxantrone + NaTDC	$2.66\ x10^{-9}$	$5.10\ x10^{-9}$	$1.85\ x10^{-9}$
NaTC	$1.61\ x10^{-9}$	$3.78\ x10^{-9}$	$1.04\ x10^{-9}$
mitoxantrone + NaTC	$2.13\ x10^{-9}$	$3.21\ x10^{-9}$	$1.05\ x10^{-9}$
SDS	$1.80\ x10^{-9}$	$1.73x10^{-9}$	$3.65\ x10^{-10}$
mitoxantrone + SDS	$2.18\ x10^{-9}$	$1.76x10^{-9}$	$3.95\ x10^{-10}$

The EPR spectra of 12-DSA present a two component feature. The main component with a slower dynamic corresponds to location of the spin probe in micelle, while the other component correspond to the free spin probe in solvent. The simulations of the EPR spectra for 12-DSA indicate that the contribution of the free specie to the EPR spectra is less than 10 %.

The analysis of the results in Tables 1 and 2 shows that the values of rotational correlational time of spin probe incorporated in micelles decrease in the following order: 12-DSA > 5-DSA > 16-DSA for both bile salts micelles and in the following order: 5-DSA > 12-DSA > 16-DSA for SDS micelles, indicating a distinct location for the spin probes in these aggregates. This behaviour is similar for both *p*H values. Also, a similar variation was previously observed for SDS, sodium cholate and sodium deoxycholate micelles [27]. This different behaviour can be explained by the different assembly of bile salts and SDS in micelles. The different behaviour of 12-DSA for both bile salt solutions was observed for erythrocyte membranes and model membranes and reflects the lipid-protein interaction more strongly than the other spin probes [27].

The general behavior of all spin probes is the faster dynamics in SDS micelles compared with NaTDC or NaTC, and this illustrates a different organization of the micelles. 12-DSA is probably located deeper in the NaTDC and NaTC micelles than 5-DSA or 16-DSA.

The variation of the rotational correlational time (τ) of spin probes incorporated in NaTDC, NaTC and SDS micelles in the presence of mitoxantrone follows the same order as in the absence of the drug at both investigated pH values. At *p*H 7.4, the presence of mitoxantrone leads to an increase of τ of 5-DSA spin probes in both bile salts and SDS micelles and this increase is higher in the case of bile salts. Similarly, τ of 12-DSA spin probe in bile salts micelles increases in the presence of mitoxantrone but it remains unchanged in the case of SDS micelles. The τ values of the 16-DSA spin probe increase for NaTDC and NaTC micelles in the presence of mitoxantrone but decrease in the case of SDS micelles. The sensitivity of τ to the presence of mitoxantrone proves that the drug induces local distinct changes in the micelles in accord with the different structure of bile salt micelles as against SDS micelles. Bile salt micelles have lower aggregation numbers and are much smaller than classical micelles due to the planar structure of molecule which has to isolate the hydrophobic part of the molecule from aqueous phase [47, 48].

At *p*H 10, the presence of mitoxantrone induces a different evolution of spin probes for the three types of micelles. The τ values of the 5-DSA spin probe increase for NaTC and SDS micelles in the presence of mitoxantrone but decrease in the case of NaTDC micelles. We also point out that NaTDC and NaTC micelles in the presence of mitoxantrone show smaller τ values for 12-DSA spin probe and τ values are unmodified in the case of SDS micelles in the presence of the drug. The τ values for the 16-DSA spin probe increase for NATDC and SDS micelles in the presence of mitoxantrone and they have the same value for NaTC micelles. The pH dependence can be related to drug charge: at *p*H 7.4 mitoxantrone has two positive charges due to the protonation of amino groups on lateral chains whereas at *p*H 10 mitoxantrone is uncharged. Also, our previous absorption results [36,42] indicated higher partition coefficients at *p*H 10 compared with *p*H 7.4, showing that the neutral form of mitoxantrone are more efficient incorporated than the protonated form of mitoxantrone.

Conclusions

In this study we aimed to evidence the changes induced by mitoxantrone drug in micellar assemblies of two bile salts and SDS using the spin probe method of ESR spectroscopy. The spin probes chosen have similar structure with fatty acid, bearing the paramagnetic moiety doxyl attached in position 5 (5-DSA), 12 (12-DSA) and 16 (16-DSA). The analysis of ESR parameters of these spin probes (the hyperfine coupling constant, a_N and rotational correlational time, τ) evidenced that mitoxantrone induces small changes in micellar assemblies, in accordance with the different micellar structure of bile salts compared to SDS. If SDS micelles are spherical assemblies, the aggregation behavior of bile salt is different are from that of conventional surfactants, and they form primary aggregates due to hydrophobic interactions between the convex surfaces of monomers and secondary aggregates due to hydrogen bonding between the hydroxyl groups. The ESR parameters are also slightly sensitive to variation of pH.

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