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Incorporation of NSAIDs in micelles: implication of structural switchover in drug–membrane interaction

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Abstract

Non-steroidal anti-inflammatory drugs (NSAIDs) of oxicam group are not only effective as anti-inflammatory agents but also show diverse functions. Their principal targets are cyclooxygenases, which are membrane-associated enzymes. To bind with the targets these drugs have to pass through the membrane and hence their interactions with biomembranes should play a major role in guiding their interactions with cyclooxygenases. Here we have studied the interactions of three NSAIDs of oxicam group viz. piroxicam, meloxicam and tenoxicam with micelles having different headgroup charges, as simple membrane mimetic systems. Spectroscopic methods have been used to understand the interaction of these drugs with Cetyl *N*,*N*,*N*-trimethyl ammonium bromide (cationic), Sodium dodecyl sulphonate (anionic) and Triton X-100 (neutral) micelles. Our results demonstrate that the environment of the drugs i.e. the nature of the micelles plays a decisive role in choosing a specific prototropic form of the drugs for incorporation. Additionally it induces a switch over or change between different prototropic forms of piroxicam, which is correlated with the change in their reactivities in presence of different surface charges, given by the change in pK_a values. These results together, indicate that in vivo, the diverse nature of biomembranes might play a significant role in choosing the particular form of oxicam NSAIDs that would be presented to their targets.

Keywords: COX-inhibitors; Oxicam drugs; Optical spectroscopy; Anisotropy; Free energy change; Chemical reactivity

1. Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) of the oxicam group are conventionally known as highly effective class of drugs against various arthritis and post operative inflammations [1]. They are also important for other therapeutic functions such as chemoprevention [2,3] and chemosuppression [4,5] in different cancer cell lines [4] and animal models [3]. Some of them are also good UV-sensitiser [6–8] and UV-protector of skin [9]. These drugs are not only important for their great therapeutic potential, but also for their interesting chemical properties by virtue of their dynamic structural features [10–14], which make them extremely sensitive to their microenvironment. They act as anti-inflammatory and analgesic agents by inhibiting cyclooxygenases, which are key enzymes involved in the inflammatory

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response of the body. They catalyse the production of inflammatory prostaglandins from dietary arachidonic acids. Cyclooxygenases principally have two isoforms viz. COX-1 and COX-2. Recently another isoform COX-3 has also been isolated [15]. All these isoforms are membrane associated proteins [1]. So the first level of interaction of these drugs are with the membranes. It is known that the oxicam drugs can exist in different prototropic forms (cationic, neutral/zwitterionic and anionic) under different physiological conditions [13]. Even though the mode of binding of the oxicam drugs are somewhat known from X-ray crystallographic data [1], it is not at all clear which structural form of these drugs bind to their targets in vivo. We have shown earlier that the principal form available for interaction is guided or selected by their microenvironment [16]. To inhibit the activity of cyclooxygenases, these drugs have to pass through the membrane and hence their interaction with membranes should play a major role in choosing the principal form of these drugs that would be presented to their targets.

In order to study the interaction of the oxicam drugs with membranes, micelles are chosen as the simplest membrane mimetic systems. In the present work, piroxicam [4-hydroxy-2-methyl-*N*-(pyridin-2-yl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide] has been chosen as a prototype NSAID belonging to the oxicam group. Three different types of micelles with varying headgroup charges were used viz. Cetyl *N*,*N*,*N*-trimethyl ammonium bromide (CTAB) with positively charged headgroup, Sodium dodecyl sulphonate (SDS) with negatively charged headgroup and Triton X-100 (TX-100) with neutral headgroup. Our investigation is extended to include two more oxicam drugs, viz. tenoxicam [4-hydroxy-2-methyl-*N*-(pyridin-2-yl)-2H-thieno (2,3-e)-1, 2 thiazine-3-carboxamide 1,1-dioxide] and meloxicam [4-hydroxy-2-methyl-*N*-(5-methyl-2-thiazolyl)-2H-1,2-benzothiazine-3-carboxamide 1,1-dioxide], which are generated by isosteric substitution of the aryl and pyridyl ring of piroxicam, respectively (Fig. 1). While piroxicam and tenoxicam are known to exist in neutral, zwitterionic and anionic forms, no evidence for zwitterionic form of meloxicam has been reported [13].

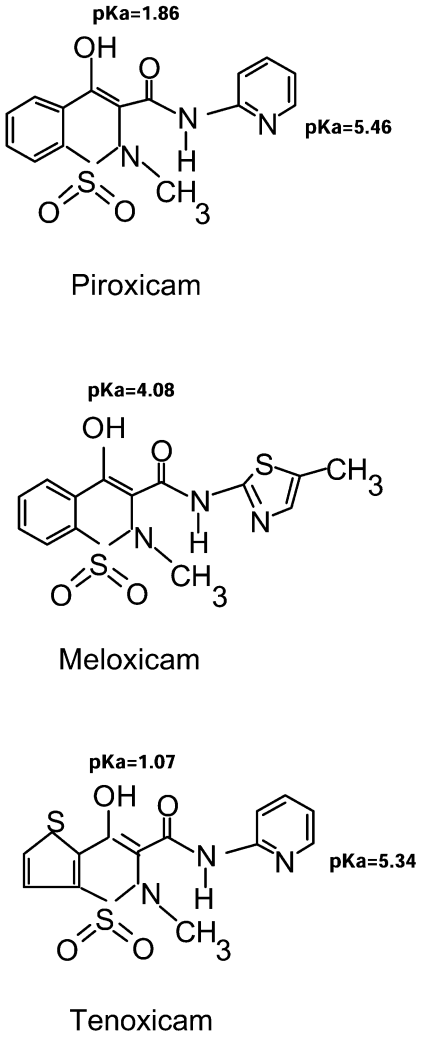

Fig. 1. Chemical structure of piroxicam, meloxicam and tenoxicam along with their pK_a values.

The incorporation of different prototropic forms of the drugs in different micelles have been studied using UV–visible absorption and steady state fluorescence spectroscopy including steady state fluorescence anisotropy measurements. Our study clearly demonstrates that the nature of micellar environment is capable of modulating the local environment of these drugs, which leads to a switch over or change between different prototropic forms as in case of piroxicam. In order to justify the switch over between two prototropic forms viz.

global neutral (neutral and/or zwitterionic) and anionic forms, we have measured the apparent pK_a values of piroxicam in presence of anionic and cationic micelles. Significant changes in pK_a values have been obtained in CTAB and SDS micelles, which indicate that drugs like piroxicam can offer a variety of chemical reactivities modulated by its environment. This assumes special significance when one consider the activity of these type of drugs in vivo, where the environments of diverse biological membranes are expected to select the species and reactivity of the drugs that will be presented to their targets. For tenoxicam and meloxicam the principal structural forms incorporated in different micelles have been also identified.

2. Experimental

Piroxicam and tenoxicam were purchased from Sigma Chemicals (USA) and meloxicam from LKT Laboratories Inc. (St Paul, MN) and were used without further purification. CTAB and SDS were purchased from Merck (Germany) and USB™, respectively. Water was distilled thrice before use. Stock solutions of concentration 0.5 mM were prepared in ethanol (Merck) and exact concentration was adjusted by triple distilled water. Each aqueous solution contains a maximum of 6% (v/v) of ethanol. We have changed the pH of the working solutions by adding dil HCl and/or dil NaOH to them. The volume of acid (HCl) added to the working solutions is exactly equal to the volume of acid that is needed to acidify a volume of water equal to the working solution to attain that particular pH. Similar procedure was carried out to make the solutions alkaline by adding NaOH. Solution at pH 5.5 indicates that no acid or alkali was added to the aqueous solutions. Samples were checked for photochemical changes during spectral scan time and no change was found. Temperature was kept constant at 298 K throughout all experiments.

We have measured the critical micellar concentration (CMC) of SDS and CTAB at different pH using pyrene as the chromophore following the environmental effect on the vibronic band intensities (3/1) of pyrene [17]. Below CMC, there are no micelles present and the pyrene fluorescence spectrum corresponds to the 3/1 ratio as that in water. However, as the detergent concentration increases above the CMC, pyrene is solubilised in the hydrophobic interior as illustrated by the increased 3/1 ratio. The micelle formation was followed by the sharp increase in the 3/1 vibronic band ratio of the fluorescence spectrum of pyrene, which corresponds to the CMC value of the surfactant. The CMC values obtained are 0.3 and 0.85 mM for CTAB and 5.5 and 8.0 mM for SDS at pH 0.55 and 5.5, respectively. We kept the surfactant concentration well above the CMC value at different pH during the determination of pK_a to ensure the presence of adequate number of micelles in the solution, so as to keep micellar effect uniform at every pH.

Absorption spectra were recorded with Shimadzu UV–visible spectrophotometer model UV2101PC. Baseline correction was done with water before recording each set of data. Fluorescence measurements were performed using a Jobin Yvon spectrofluorimeter model Fluoromax-3. All emission spectra were corrected for instrument response at each wavelength. A 2×10 mm^2 path length quartz cell was used for all fluorescence measurements to avoid any blue edge distortion of the spectrum due to inner filter effect [18]. Concentration of the drug was measured from Lambert Beer's Law, as the extinction coefficients are known at the characteristic wavelength of the global neutral and anionic form. Quantum yield of a particular sample was measured at least three times using quinine sulphate in 0.1 M H_2SO_4 as quantum yield standard [18]. Fluorescence anisotropy (r) was measured using the standard equation [18]:

$$r=(I_{VV}-GI_{VH})/(I_{VV}+2GI_{VH}),$$

where $G=I_{HV}/I_{HH}$ and I_{VV}, vertically polarised excitation and vertically polarised emission; I_{VH}, vertically polarised excitation and horizontally polarised emission; I_{HH}, horizontally polarised excitation and horizontally polarised emission; I_{HV}, horizontally polarised excitation and vertically polarised emission.

All measurements were done with freshly prepared samples.

3. Results and discussion

3.1. Interactions of piroxicam with micelles

NSAIDs of oxicam group can exist in different prototropic forms i.e. cationic, global neutral (neutral and/or zwitterionic) and anionic forms in different physiological conditions [13]. These prototropic forms are extremely sensitive to their microenvironment [16]. In this present study we have characterized the interaction of piroxicam with micelles of different headgroup charges and have extended it to include other oxicams like meloxicam and tenoxicam. From the pK_a values (Fig. 1) it is evident that at pH 5.5 the anionic form of piroxicam should predominate over its cationic and global neutral counterpart. The ground state electronic transitions of different prototropic forms of these drugs have been identified by us and for the anionic form of piroxicam it is n,π^* (data not shown). Fig. 2a shows the shift of the absorption maximum with increasing concentration of CTAB at pH 5.5. The absorption maximum of anionic form of piroxicam is at 353 nm in water. This peak is gradually red shifted in the pre micellar concentration of surfactant until it reaches 363 nm around CMC of CTAB (0.8 mM at pH 5.5). Increase in CTAB concentration beyond CMC results in no further red shift of the absorption maximum. For a molecule showing n,π^* transition, a red shift in the absorption maximum occurs when it goes from a polar to a non-polar environment. This 10 nm red shift of absorption maximum indicates that the anionic form of piroxicam is facing a more non-polar environment, which means that the anionic form of piroxicam is being incorporated in the CTAB micelles. The fluorescence quantum yield of the anionic species is higher in non-polar environment as compared to aqueous environment (quantum yields in water and butanol are 3.52×10^{-4} and 1.12×10^{-2}, respectively). The increase of quantum yield with increase in CTAB concentration (Fig. 2b) also supports that the anionic form of piroxicam is being incorporated into the CTAB micelles. It should be mentioned that at pH 5.5, besides the predominant anionic form of piroxicam, the global neutral form could also exist. The absorption maximum for the global neutral form of piroxicam is approximately 330 nm [16]. Increase in the rigidity of the surrounding environment of a chromophore result in an increase in the fluorescence anisotropy value monitored at an excitation wavelength characteristic of that chromophore. We have monitored the fluorescence anisotropy with increasing concentration of CTAB both at 363 and 323 nm, which are the excitation maxima of anionic and global neutral form of piroxicam, respectively. Whereas, the fluorescence anisotropy value at 363 nm increases with increasing concentration of CTAB (Fig. 2c) and saturates approximately 0.8 mM, the CMC of CTAB, the anisotropy values of piroxicam at 323 nm are randomly distributed (data not shown). From this it is clear that only the anionic form is incorporated into the positively charged CTAB micelles, resulting in the drug facing a more rigid environment compared to the aqueous phase, whereas the global neutral form remains in the bulk water.

In presence of SDS, (negatively charged micelles), the absorption maximum does not show any shift at pH 5.5 with increasing concentration of SDS. This is because the predominant population at this pH is the anionic form, which is expected to be repelled by the negatively charged headgroups of anionic micelles. In order to see if the global neutral form can be incorporated in anionic micelles, the pH of the solution was adjusted to 3.8 to have adequate amount of global neutral form of piroxicam in solution. Fig. 3a shows a plot of the absorption maximum of piroxicam with increasing concentration of SDS. Initially, without added surfactants in solution, the absorption maximum was at 353 nm, which means that even at this pH the anionic species was predominating over the global neutral form. Increasing SDS concentration resulted in a gradual change of absorption maxima from 353 to 334 nm (Fig. 3a). The extinction coefficient of the global neutral form is higher than that of the anionic form in both polar and non-polar environments. The extinction coefficients in water of the global neu-

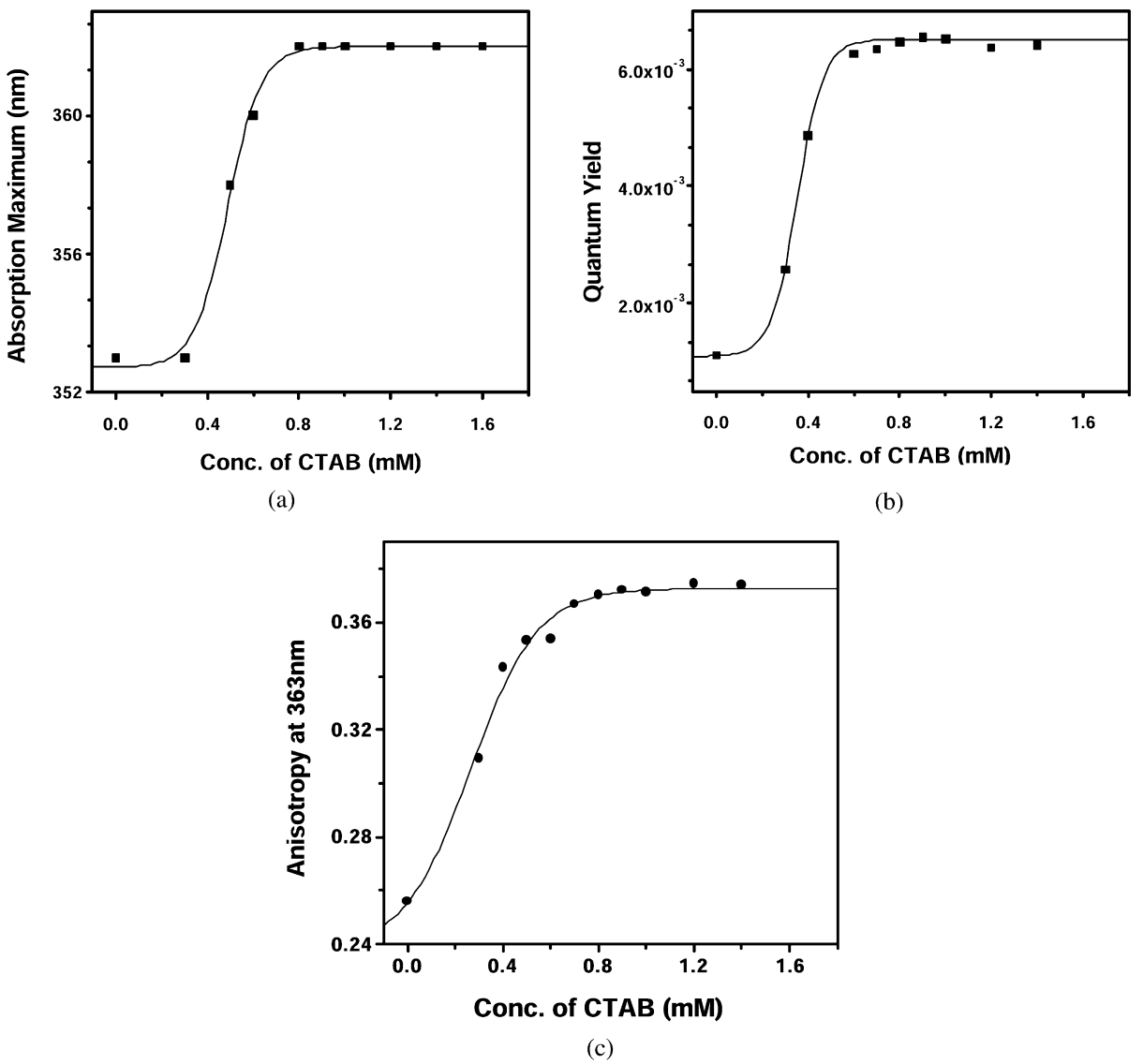

Fig. 2. Plot of (a) absorption maximum; (b) quantum yield; (c) anisotropy at 363 nm of piroxicam vs. concentration of CTAB. The piroxicam concentration was kept constant at 30 μM and pH at 5.5.

tral and anionic forms are 3.521×10^4 and 2.434×10^4 $M^{-1}\,cm^{-1}$, respectively, whereas in butanol they are 2.503×10^4 and 1.475×10^4 $M^{-1}\,cm^{-1}$, respectively. The change in the absorption maximum from 354 to 334 nm indicates that the neutral form of piroxicam is incorporated in the anionic micelles. Since the extinction coefficients are not very different between the two forms the question arises as to why we see the shift in the absorption maximum? This can be explained in terms of a switch over or change from anionic to global neutral forms as discussed in detail in the next section. In this case also, the saturation starts from 6 mM of SDS, which is the CMC of SDS at pH 3.8. The global neutral form of piroxicam has a higher quantum yield in the non-polar

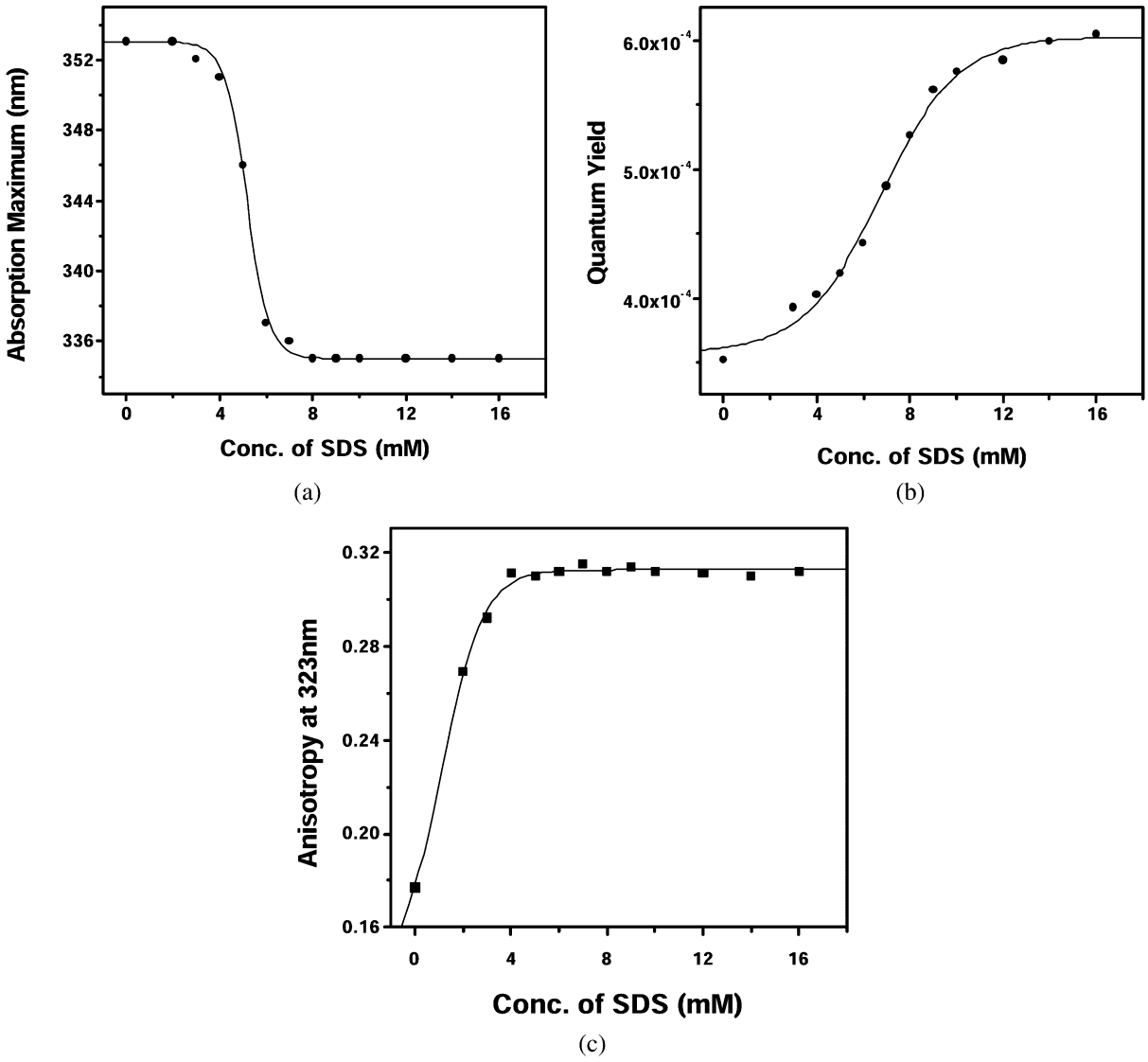

Fig. 3. Plot of (a) absorption maximum; (b) quantum yield; (c) anisotropy at 323 nm of piroxicam vs. concentration of SDS. The piroxicam concentration was 30 μM at pH 3.8.

environment than that in the polar environment (quantum yield in water and butanol is 1.11×10^{-3} and 3.19×10^{-2}). So the increase of quantum yield of the neutral form with increasing SDS concentration also indicates the incorporation of the global neutral form into the micelles (Fig. 3b). It should be noted that in this case, the increase in fluorescence anisotropy is observed at 323 nm with increasing concentration of SDS (Fig. 3c) which confirms the incorporation of global neutral form of piroxicam in the negatively charged micelles, whereas the fluorescence anisotropy value at 363 nm, characteristic of the anionic form, is randomly distributed. This shows that the anionic form was left behind in the bulk aqueous phase.

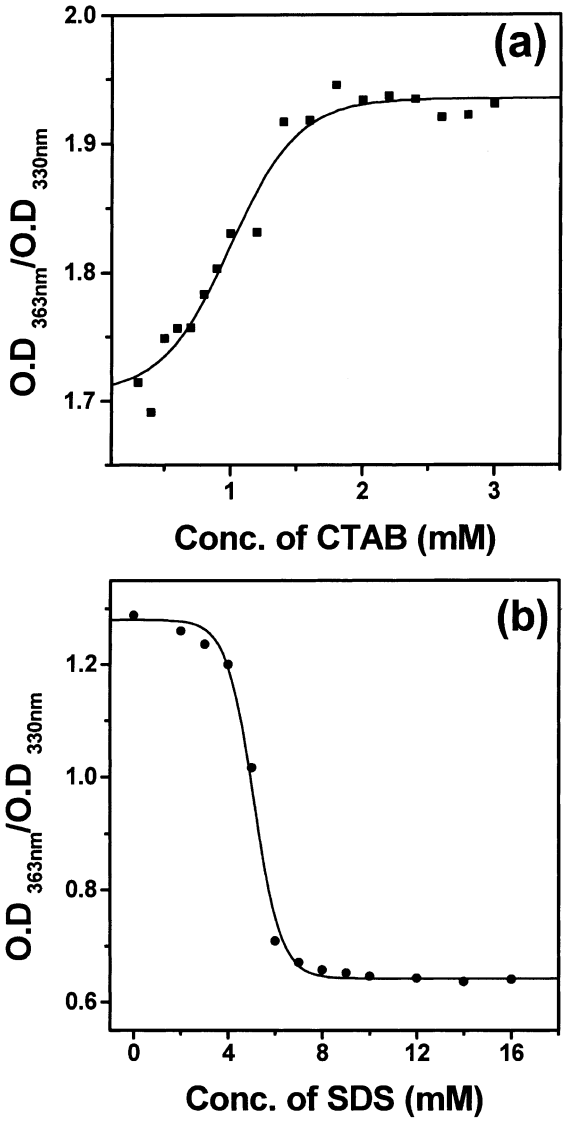

Fig. 4. Plot of O.D. ratio at 363 nm to 330 nm, the absorption maxima of anionic and global neutral form of piroxicam against (a) concentration of CTAB at pH 5.5 and (b) concentration of SDS at pH 3.8. The drug concentration was kept at 30 μM.

3.2. Switch over between different prototropic forms of piroxicam depending on the nature of micelles

Fig. 4a shows the plot of optical density ratio at 363 nm and 330 nm (the absorption maxima of anionic and global neutral forms, respectively) with increasing CTAB concentration. Interestingly it is found that this ratio increases with the concentration of CTAB, even though the extinction coefficient of the global neutral form is higher than that of the anionic form as mentioned before. This means that the amount of anionic form is increased in the presence of positively charged CTAB. On the other hand the optical density ratio at 330 nm to 363 nm increases with the concentration of SDS (Fig. 4b), which indicates that the amount of the global neutral form is increased in presence of negatively charged micelles. Let us consider the following equilibrium

$$N \rightleftarrows A$$

where 'N' represents the global neutral and 'A' the anionic form of piroxicam.

Since the micellar phase is spectroscopically silent, the effect of micellar equilibrium is indirectly reflected in the changes in spectral properties of the drug molecule itself. The equilibrium constant (K) is given by

$$K = [A]/[N]$$

where [A] is the concentration of the anionic form and the [N] is the concentration of the global neutral form.

The change in free energy (ΔG) is determined from the equation

$$\Delta G = -RT \ln K$$

Change in ΔG means the difference between ΔG values at the highest concentration of surfactant and without added surfactant. Considering the above equilibrium, the ΔG value becomes more negative in presence of CTAB (Fig. 5a) and positive in presence of SDS (Fig. 5b). A negative ΔG value means that the neutral to anion formation is favoured in presence of cationic micelles CTAB, whereas ΔG value positive means the global neutral to anion conversion is disfavoured in presence of SDS micelles. The maximum change in the ΔG value in presence of CTAB is -0.3 kJ/mol and that in presence of SDS is 1.7 kJ/mol.

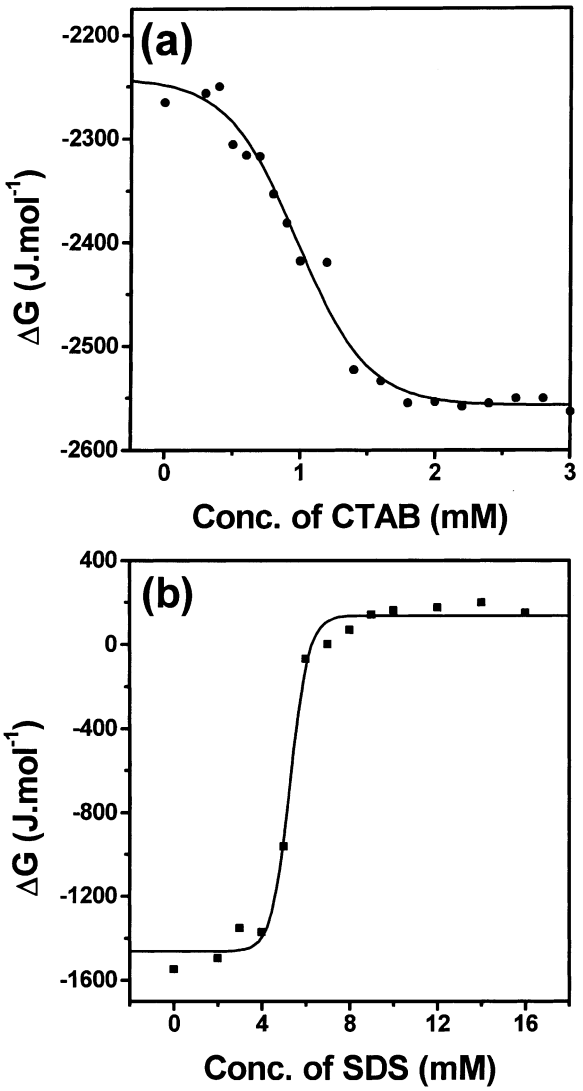

Fig. 5. Dependence of ΔG (kJ/mol) with concentration of (a) CTAB at pH 5.5 and (b) SDS at pH 3.8 for 30 μM piroxicam.

In order to find out the underlying cause that results in the switch over between different prototropic forms of piroxicam, we have measured the apparent pK_a values in presence of CTAB and SDS micelles. The relative absorption of the anion/global neutral forms was recorded with increasing pH, and the corresponding concentration ratio of the anionic/global neutral forms is plotted against pH in Fig. 6. The pK_a values in absence of any surfactant, in presence of CTAB and in presence of SDS are 2.60, 2.72 and 3.53, respectively. So the switch over or change between different forms observed in presence of oppositely charged micelles is due to the change in the apparent pK_a values. Change in ΔG value is less in presence of CTAB as the change of pK_a of piroxicam is small, whereas this change is larger in case of SDS corresponding to a larger shift in the pK_a in presence of SDS micelles. It should be noted that we have kept the micellar concentration well above the CMC values in the full range of pH variation studies. As it has been mentioned in the experimental section, the CMC of both CTAB and SDS in different pH were measured using pyrene as the chromophore according to standard technique. The concentration of CTAB was kept at 1.5 mM and for SDS it was 15 mM.

The possible reasons behind the change of pK_a values are as follows: (a) ionic interaction between the surfactant and the different prototropic species of piroxicam, (b) the hydrophobic interaction between the non-polar region of the micelles and

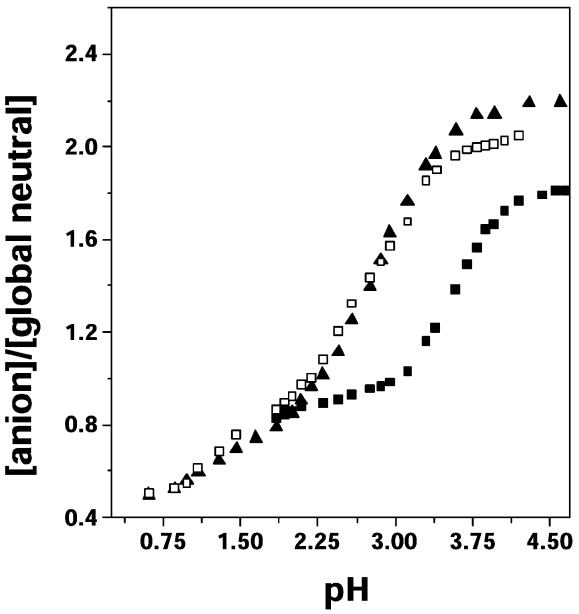

Fig. 6. Trace of (anion)/(neutral) of piroxicam without micelles (□), in presence of CTAB (▲) and in presence of SDS (■) with varying pH.

the drug. This is dependent both on its location in the microheterogeneous environment of the micellar phase as well as the particular orientation of the drug molecule in the pseudomicellar domains. The pK_a shift affected by the cationic micelles has the same direction as that of anionic micelles (Fig. 6). This shows that the change in pK_a is not only due to ionic interactions, but points to a more complex mechanism that is difficult to isolate at this stage. Large changes in pK_a values have also been observed in case of a single dopant in micelles induced by additional sol–gel entrapment [19]. The authors have attributed their observed pK_a shifts not to inherent changes in the equilibrium constant, but to external conditions which are needed to compensate for local effects within the sol–gel cage. In our work, since there is no such additional entrapment within a sol–gel cage, such large changes in pK_a values are not observed. The change in apparent pK_a could reflect both local effects prompted by surface charge of the micelles and by inherent changes in the chemical reactivities of the drugs. By local effects we mean that the local pH near the surface of a charged micelle will be different from that of the bulk. Hence the apparent pK_a of the drugs will be shifted as the bulk pH is measuring a different value than the local pH near the surface. However, at this stage it cannot be isolated whether for these drugs, inherent changes in chemical reactivities also contribute to the change in pK_a values. In this connection, it should be mentioned that in an earlier work [16] we have demonstrated the extreme sensitivity of oxicam drugs to their microenvironment. Even small quantity of water can select the predominant prototropic form in solution though the pH of the solvent was not changed. This could imply that microenvironment might also induce changes in chemical reactivity in the oxicam drugs. The above studies were also carried out in presence of TX-100 micelles with neutral headgroup. Increasing concentration of TX-100 did not affect the spectral parameters of the drug. This indicates that the drug does not interact with TX-100 and are left in the bulk aqueous phase. The same is also true for other oxicam drugs viz. tenoxicam and meloxicam. From our results it is clear that the nature of micelles not only select a particular prototropic form of the drug that would be incorporated in it, but also modulate the apparent pK_a values resulting in a switch over between different prototropic forms.

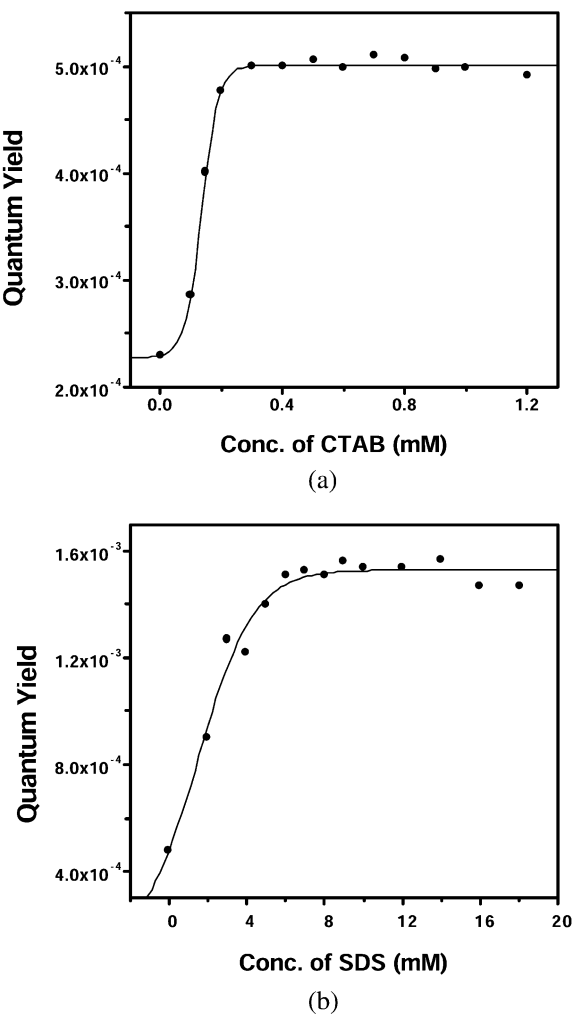

Fig. 7. Plot of quantum yield of meloxicam vs. (a) concentration of CTAB at pH 10.0 and (b) concentration of SDS at pH 2.5. The concentration of meloxicam was kept at 30 μM.

3.3. Interactions of other oxicam drugs with micelles

We have also studied the interaction of two other oxicam drugs viz. tenoxicam and meloxicam in different micellar systems. The experiments

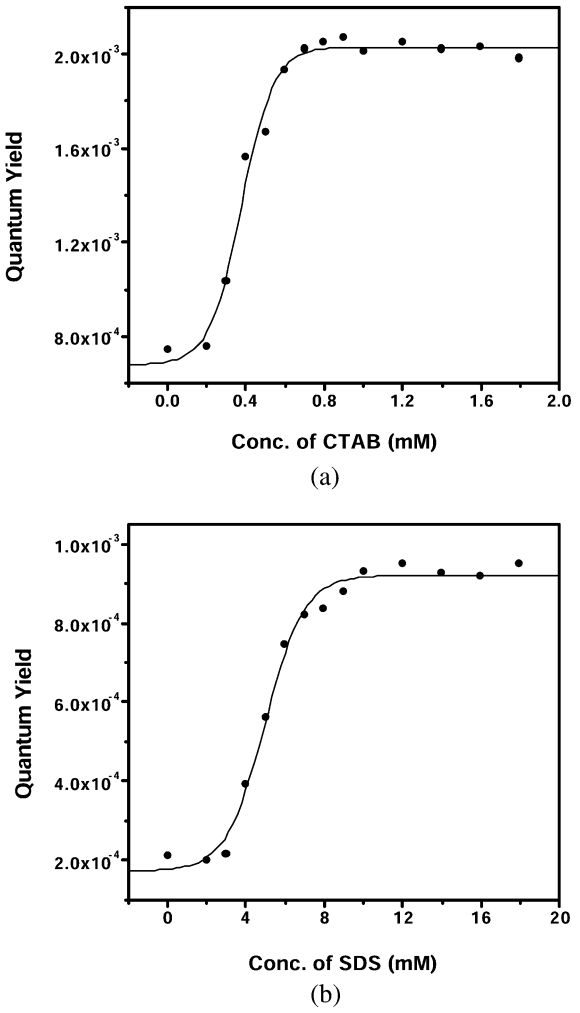

Fig. 8. Plot of quantum yield of tenoxicam vs. (a) concentration of CTAB at pH 10.0 and (b) concentration of SDS at pH 2.5. The concentration of tenoxicam was kept at 30 μM.

were carried out at pH 2.5 and 8.0 to study the incorporation of the global neutral form and the anionic form in the micelles, respectively. Fig. 7a and Fig. 8a show that the quantum yields of the anionic forms of meloxicam and tenoxicam increase with increasing concentration of CTAB at pH 8.0. Here also the quantum yield of anion is higher in non-polar environment than in polar environment (quantum yield of the anionic form meloxicam in water and butanol are 2.23×10^{-4} and 1.14×10^{-2}, respectively, and quantum yield of the anionic form of tenoxicam in water and butanol are 1.17×10^{-4} and 1.12×10^{-2}, respectively). The enhancement of quantum yield in presence of CTAB indicates that the anionic forms of these two drugs are incorporated into the cationic micelles. Fig. 7b and Fig. 8b show that the quantum yield of the neutral form of meloxicam and tenoxicam increases with increasing concentration of SDS at pH 2.5. The quantum yield of the neutral form of meloxicam and tenoxicam is higher in the non-polar environment than in the polar one (quantum yield of the neutral form of meloxicam in water and butanol are 4.78×10^{-4} and 2.31×10^{-2}, respectively, and quantum yield of the global neutral form of tenoxicam in water and butanol are 2.12×10^{-4} and 1.69×10^{-2}, respectively). Hence it is clear that like piroxicam, the neutral forms of both meloxicam and tenoxicam are incorporated in the negatively charged micelles, SDS, whereas the anionic species of these two drugs are incorporated in cationic micelles, CTAB.

4. Concluding remarks

The extreme sensitivity of the oxicam group of NSAIDs to their microenvironment is also reflected in their interactions with micelles. The nature or charge of the micellar headgroup guides which forms of piroxicam, tenoxicam and meloxicam would be incorporated in the micelles. The anionic forms of the drugs interact with cationic micelles and the neutral forms with anionic micelles. Not only the nature of the micelles select a particular form of the drugs for incorporation, but it also induces switch over between different prototropic forms as in case of piroxicam. This switch over could be correlated to changes in apparent pK_a values. Such modulation of chemical properties by their microenvironments might play an important role in vivo, where the drug molecules face diverse environments and hence their interactions with other biomolecules could also vary depending on their exposed surroundings. Our study implies that the nature of the biomembranes might play a decisive role in selecting the prototropic form and reactivity of these oxicam drugs that would be finally presented to their targets.

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