Interactions Between Oxicams and Membrane Bilayers: an Explanation for Their Different COX Selectivity

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Abstract: Meloxicam was launched as a major new NSAID for the treatment of arthritis following extensive published research confirming its selectivity for COX-2. Several studies proposed possible explanations for its effectiveness and superior safety profile. The proposed theories included chemical structural relationships between meloxicam and other effective NSAIDs with low gastrointestinal toxic effects. However, other oxicams have similar chemical groups, but despite this, are not considered COX-2 selective drugs and exhibit less gastric tolerance. Hence, the aim of this work was to investigate the interactions between oxicams and biomembrane models as it could influence their resorption from the upper gastrointestinal tract and may affect their local gastromucosal tolerability.

The partition of oxicams within membranes was determined by calculating their partition coefficients between liposomes and water. Moreover, their location within the bilayer was determined by fluorescence quenching. Finally, zeta-potential measurements were made to complete the information about the binding behaviour of the oxicams and steady-state anisotropy measurements were made to determine their induced perturbation in membrane structure. These studies proved that, in spite of structural similarities, oxicams present different interactions with membranes making possible a virtual division of the class in two groups. Tenoxicam and piroxicam known as COX-1 inhibitors demonstrated higher partition capacity in liposomes/water systems together with a smaller ability to change the membrane fluidity and surface potential. In contrast lornoxicam and meloxicam, which demonstrated activity against COX-2, have revealed smaller partition capacity in liposomes/water systems together with a higher ability to change the membrane fluidity and surface potential.

INTRODUCTION

Non-steroidal anti- inflammatory drugs, NSAIDs, are a chemically heterogeneous group of agents possessing a common property of being endogenous prostaglandins (PGs) synthesis inhibitors *via* inhibition of the cyclooxygenase (COX) enzyme, an action that has been generally accepted as the principal mechanism by which these drugs act [1-4]. Recently, it has been established that COX enzyme has two isoforms (constitutive COX-1 and the inducible COX-2) and current thinking suggests that inhibition of COX-1 is involved directly in the side effects of NSAIDs while the therapeutically desirable effects come from inhibition of COX-2 [5, 6]. Nevertheless, the notion that a highly selective COX-2 inhibitor will alone lead to low gastroulcerogenicity is open to question and no clear correlation has been found between the inhibition of COX-1 activity by NSAIDs and damaging effects on gastrointestinal function [7]. In fact, there are several currently available NSAIDs that have appreciable COX-1–inhibitory activity as well as COX-2 effects that have relatively low gastrointestinal ulcerogenicity [8, 9].

Another intriguing aspect about NSAIDs' varying degrees of toxicity and anti-inflammatory activity is that these effects occur not only in structurally unrelated compounds, but also within homologous series of NSAID "families". NSAIDs of the oxicam group are conventionally known as a highly effective class of drugs against various arthritis and postoperative inflammations [10], but despite their similar chemical structures, they have different biological effects. Indeed, in addition to high anti-inflammatory efficacy, meloxicam, which is a member of oxicam class, appears to have low ulcerogenic potency and exhibits less gastric and local tissue irritation in comparison to other NSAIDs, including other oxicams. Many authors have tried to establish what factors may account for the apparent low ulcerogenicity of meloxicam [11, 12]. Some authors considered that the good tolerability profile may be explained by the proved ability of meloxicam to preferentially inhibit COX-2 [13-15]. Other authors proposed that the chemical structures of the COX-2 inhibitors (including meloxicam) have sulfa moieties [16] that clearly distinguish them from conventional carboxylic acids or ketoenolic acids that characterize non selective COX-2 inhibitors (Fig. **1**).

This chemical difference would permit the inhibition of the COX-2 enzyme possibly exploiting increased flexibility of the inner shell of the roof of the enzyme [17]. Also, because of the sulfa moieties present in COX-2–selective NSAIDs, some authors proposed that these compounds had much higher pKa values than are normally encountered in COX-1/COX-2 inhibitors and this might contribute for the low local mucosal irritancy observed with these drugs inde-

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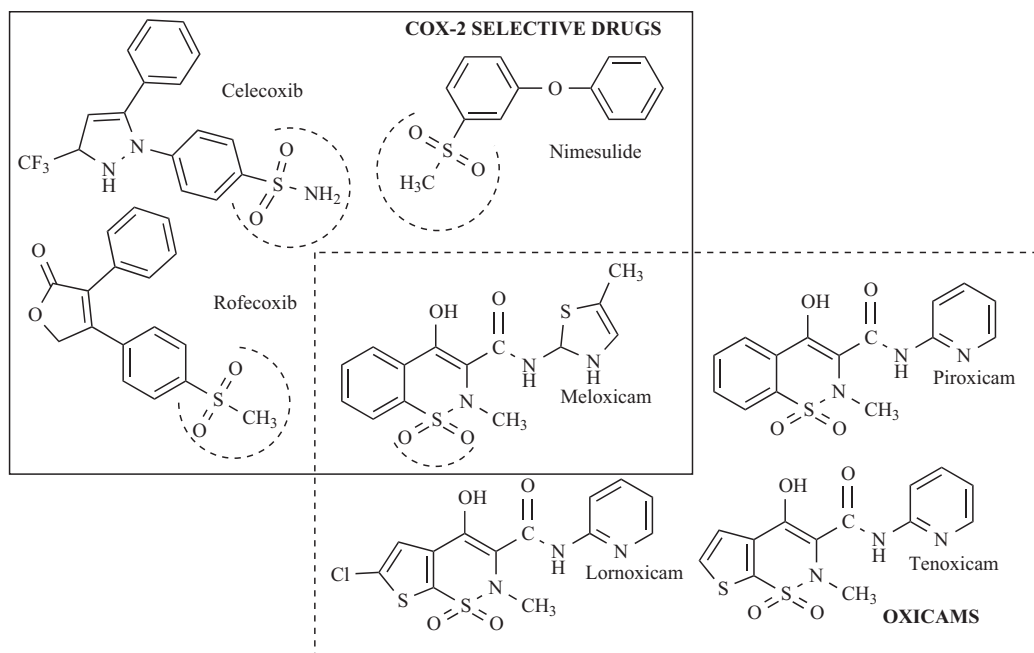
Fig. (1). Chemistry of COX-2 selective drugs compared with oxicams.

pendent of their COX-2 selectivity [18, 19]. Still, despite these suggested theories, for unexplained reasons the other oxicams which have the same sulfa-moieties encountered for meloxicam, Fig. **1**, are not considered COX-2 selective drugs and exhibit less gastric tolerance [14]. Consequently, it is clear that the pathogenesis of NSAID-induced gastrointestinal damage is complex, and there are also other important non-prostaglandin-mediated effects to the damage, including the "topical" effect, which may result from an interaction between NSAIDs and surface phospholipids of the gastrointestinal membranes [20-23], together with the systemic and local effects caused by inhibition of membrane bound enzyme COX. Although the precise details concerning the explanation of NSAIDs toxic and therapeutic actions has yet to be fully established, there is consensual evidence that their lipid affinity is of major significance. Indeed, depending on their hydrolipophilic character, NSAIDs can be distributed between the membrane and the aqueous phases and this distribution determines their concentration in each phase and thereby controls the extents of their penetration into the membrane and/or interactions with phospholipids or other membrane components such as COX enzymes which are embedded in the lipid bilayers.

Therefore the aim of this work was to investigate the interactions between four oxicams (meloxicam, lornoxicam, tenoxicam and piroxicam) and biomembrane models once that it might be relevant for understanding the apparently contrasting behaviour of meloxicam.

Firstly, the partition of oxicams within membranes was determined by calculating their partition coefficients (K_p) between lipid bilayers of egg yolk phosphatidylcholine (EPC) unilamellar liposomes (LUVs) and water, using two different experimental techniques: derivative spectropho-

tometry and fluorescence quenching. Moreover, the location of this group of NSAIDs within the bilayer was also determined by fluorescence quenching using a set of *n*-(9-anthroyloxy) fatty acid probes (*n*=2, 6, 9 and 12), which contain the same fluorescent group, but bounded at different positions of an alkyl chain (bound to C_2 in 2-AS; to C_6 in 6-AS; to C_9 in 9-AS and to C_{12} in 12-AS). Thus the fluorophores report the environment at a graded series of depths within the host lipid structure making possible a precise mapping of the NSAIDs according to differences in its quenching efficiencies [24].

Additionally, zeta-potential measurements were made, as it is important to know the influence of the drugs in the membrane surface potential, which gives an indication of the type of interactions that exist between drugs and the lipid bilayer surface.

Finally, as modifications of physical characteristics of the membrane lipid bilayer, by means of membrane fluidity may lead to disruption of phospholipid layers, and this may underlie the topical irritancy in the gastrointestinal mucosa presented by NSAIDs [25], steady-state anisotropy measurements were used to determine the oxicams' induced perturbation in membrane structure at different depths of the bilayer. In these studies, the same set of *n*-AS probes were used, as they offer the possibility of studying the fluidity gradient in model membranes [26-28].

EXPERIMENTAL

Chemicals

The anti-inflammatory drugs (meloxicam, piroxicam and tenoxicam), EPC and ($\pm$) -12-(9-anthroyloxy)-stearic acid (12-AS) were from Sigma; the other probes, ($\pm$) -2-(9-anthro-

yloxy)-stearic acid (2-AS), (±) -6-(9-anthroyloxy)-stearic acid (6-AS) and (±) -9-(9-anthroyloxy)-stearic acid (9-AS) were from Molecular Probes; all were used without additional purification. Lornoxicam was kindly provided by Euro-Labor pharmaceutics.

All other chemicals were from Merck (p.a). Solutions were prepared with double-deionised water (condutivity less than 0.1 μS cm^{-1}), and for all solutions studied the ionic strength was adjusted to 0.1 M with NaCl.

Preparation and Fluorescence Labelling of Liposomes

Liposomes were prepared by evaporation to dryness of a lipid solution in chloroform/methanol (9:1) with a stream of nitrogen; the lipid film was then left under vacuum overnight to remove traces of the organic solvents. The resultant dried lipid film was dispersed into a buffer (Hepes: 10 mM, I=0.1 M, pH 7.4) and the mixture was vortexed to yield multi-lamellar vesicles. Lipid suspensions were then equilibrated at 25.0 $\pm$ 0.1 ºC for 30 min and were further extruded 10 times through polycarbonate filters with a pore diameter of 100 nm to form LUVs [29]. EPC concentration in vesicle suspensions was determined by phosphate analysis using the phosphomolybdate method [30].

The fluorescence probes were dissolved in ethanol and added to a suspension of pre-formed liposomes with gentle mixing. The ratio of lipid to probe was always greater than 100:1 to prevent changes in the structure of the liposome membranes [31], and to ensure complete incorporation of the probe in the lipid bilayer the suspensions were left for 30 minutes.

Determination of Partition Coefficients by Derivative Spectrophotometry and Fluorescence Quenching

The oxicams' partition coefficients values (K_p) were determined in LUVs suspensions at pH 7.4. In the derivative spectrophotometry studies, buffered solution (Hepes: 10 mM, I = 0.1 M, pH 7.4) of NSAIDs were added to liposome suspensions prepared as described above; the final drug concentration was 22 μM for Lornoxicam; 47 μM for Meloxicam; 30 μM for Tenoxicam and Piroxicam while the EPC concentration ranged from 0 to 1000 μM. The resulting suspensions were incubated in the dark for 30 min. After equilibration, the absorption spectra were recorded at 25.0+0.1ºC with a Perkin Elmer Lambda 45 UV/VIS spectrophotometer, in the 200-400 nm range and using quartz cells with a 1-cm path length.

In fluorescence quenching studies, buffered solutions (Hepes: 10mM, I=0.1 M, pH 7.4) of NSAIDs were added to liposomes prepared with the 12-AS probe as described above. Final concentration range of EPC was from 50 to 1000 μM. For each EPC concentration, the oxicams were added in final concentration ranges: lornoxicam and meloxicam from 0 to 60 μM; tenoxicam and piroxicam from 0 to 50 μM. The resulting suspensions were incubated in the dark for 2 h. Fluorescence intensity measurements were made using a Perkin-Elmer LS 50B steady-state fluorescence spectrometer equipped with a constant-temperature cell holder. The sample was contained in 1 cm path length cuvette that has been

flushed with nitrogen and capped. All data was recorded at 25.0 $\pm$ 0.1ºC. Excitation wavelength was set to 381 nm and emission wavelength to 446 nm. Fluorescence intensity values were corrected for absorbance of the quencher (NSAID) at the excitation wavelength [32].

Drug Location Studies by Fluorescence Quenching

Quenching studies were performed at pH 7.4 in LUV suspensions incorporating the *n*-AS fluorescence probes, and for which the EPC concentration was approximately 500 μM. Buffered solutions (Hepes: 10mM, I=0.1 M, pH 7.4) of NSAIDs were then added to the liposomes and final drug concentrations were in the range 0-60 μM for lornoxicam and meloxicam and 0-40 μM for tenoxicam and piroxicam. The resulting suspensions were incubated in the dark for 2 h. Fluorescence intensity measurements were made using a Perkin-Elmer LS 50B steady-state fluorescence spectrometer equipped with a constant-temperature cell holder. The sample was contained in 1 cm path length cuvette that has been flushed with nitrogen and capped. All data was recorded at 25.0 $\pm$ 0.1ºC. Excitation wavelength was set to 384 nm and emission wavelength to 446 nm for 12-AS; to 448 nm for 9-AS; to 449 nm for 6-AS and to 452 nm for 2-AS. Fluorescence intensity values were corrected for absorbance of the quencher (NSAID) at the excitation wavelength [32].

Membrane Fluidity Studies by Fluorescence Anisotropy Measurements

Steady-state anisotropy measurements (r_{ss}) were performed in the same Perkin Elmer LS-50 spectrofluorimeter with polarizers inserted (excitation/emission wavelengths and slits were set as described). The sample was excited with vertically polarized light and fluorescence intensities were recorded with the analysing polarizer oriented parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the excitation polarizer. These values were used to calculate steady-state anisotropy (r_{ss}) [24]:

$$r_{ss} = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}} \qquad (1)$$

where G is an instrumental correction factor [24]. Readings were taken with an integration time of 50 s.

Zeta-Potential Measurements

The zeta-potential (ζ-potential) values of the vesicles, with and without incorporated drug, were determined at pH 7.4 (Hepes buffer), at 25.0 $\pm$ 0.1 ºC, by quasi-elastic light scattering analysis using a ZET 5104 cell in a Malvern Zeta-Sizer 5000, with a 90º scattering angle. Lipid concentration was kept constant at 500 μM. The NSAIDS were added to obtain a concentration range from 0 to 300 μM for lornoxicam and 0 to 1000 μM for meloxicam. The very low solubility of tenoxicam and piroxicam required the use of $(CH_3)_2SO$:water solutions (1:99 v/v) to obtain a concentration range from 0 to 400 μM for tenoxicam and 0 to 300 μM for piroxicam; with this solvent composition no membrane damage occurs [33]. Reference solutions without drug were prepared to ensure that ζ-potential values were not modified by the presence of DMSO.

RESULTS

Determination of Partition Coefficients by Derivative Spectrophotometry

Spectrophotometric analysis of heterogeneous media containing vesicles is hampered by the strong spectral interference caused by light scattering, which must be eliminated before partition coefficients can be calculated. The use of second derivative spectrophotometry allowed for total elimination of background signal effects arising from light scattered by lipid vesicles, without the need of separation techniques that may disturb equilibrium states [34-36].

Considering the definition of the molar partition coefficient of a drug between lipid bilayer vesicles suspensions and aqueous solution (K_p) [37] and the observation that the absorbance of a drug in a suspension containing liposomes with different lipid concentrations [*Lipid*] is: $Abs_f = Abs_m + Abs_a$, where Abs_m is the absorbance calculated assuming that all drug is membrane bound and Abs_a is the absorbance of the drug in buffer solution without lipid, then the following expression can be obtained [34, 35]:

$$Abs_f = Abs_a + \frac{\left(Abs_m - Abs_a\right)K_p\left[Lipid\right]V_\phi}{1 + K_p\left[Lipid\right]V_\phi} \qquad (2)$$

A formally identical expression can be used for derivative spectroscopy, but with *Abs* replaced by $D=(d^n Abs)/(d\lambda^n)$. The partition coefficients are calculated by fitting equation (2) to the experimental data (D_f versus [*Lipid*]), using a non-linear regression method, where the adjustable parameter is K_p. For EPC, $V_\phi = 0.688$ (in $Lmol^{-1}$), and the mean molecular weight is 700 [38].

Table **1** shows the mean values obtained for K_p for each oxicam drug.

To make sure that the background signals are effectively eliminated, the Kp values were never calculated at wavelengths (λ) where the scattering is high (λ chosen are indicated in order 377 nm; 415 nm; 272 nm; 289 nm for lornoxicam, meloxicam, tenoxicam and piroxicam, respectively). As an example, Fig. **2** shows the second derivative spectra cal-
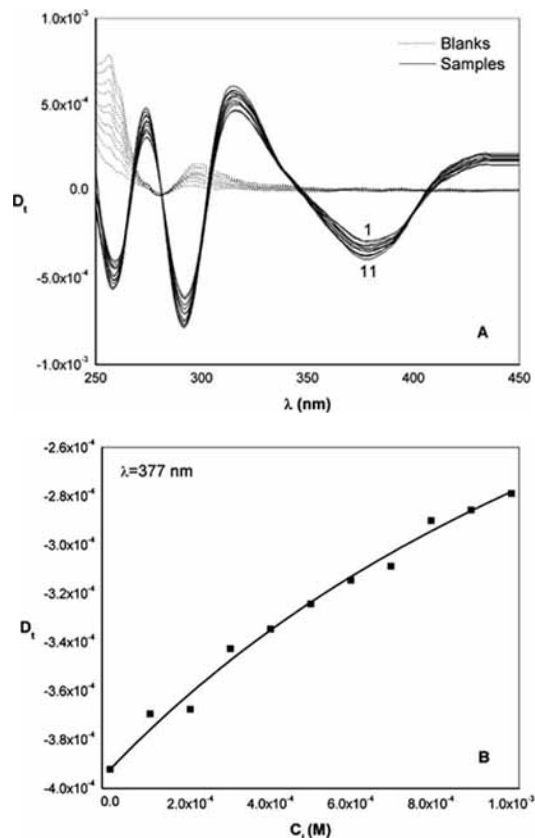

Fig. (2). (**A**) Second derivative spectra of lornoxicam at different EPC concentrations (0; 103; 198; 295; 302; 400; 498; 604; 807; 905 and 1000 μM). (**B**) Second-derivative spectrophotometric data at $\lambda= 377$ nm for lornoxicam at different EPC concentrations (0; 103; 198; 295; 302; 400; 498; 604; 807; 905 and 1000 μM). The curve represents the best fit by equation 2.

culated from the recorded spectra of lornoxicam after blank subtraction and the correspondent fit, which permitted the calculation of K_p.

Table 1. Partition Coefficients (Adimensional) for Oxicams in EPC Unilamellar Liposomes (LUV) Obtained by Derivative Spectrophotometry and Fluorescence Quenching Studies

Oxicams	Kp		Ksv
	Derivative spectrophotometry	Fluorescence quenching	
Meloxicam	685±70	573±106	92±24
Lornoxicam	493±81	450±40	222±20
Tenoxicam	2300±200	2320±90	30±0.67
Piroxicam	2700±100	2680±190	7.3±0.30

[a] The reported values are the mean of at least three independent measurements; the error that affects each value is the standard deviation.

Determination of Partition Coefficients by Fluorescence Quenching

Quenching of fluorescence can be described by the classic Stern-Volmer equation [24, 39-47]. However, when the oxicams (quencher) are distributed between membrane and aqueous phase and only the total quencher concentration is known, the Stern-Volmer equation can be written as:

$$\frac{I_0}{I} = 1 + K_{SV}^{app}[Q]_T \qquad (3)$$

where I_0 and I are, respectively, the corrected fluorescence intensity of the fluorophores (*n*-AS probe) in the absence and presence of the drug; $[Q]_T$ is the total quencher concentration (given by the sum of concentrations in aqueous and membrane phases) and the Stern-Volmer constant, K_{sv}, is replaced by the apparent Stern-Volmer constant, K_{SV}^{app} [46]. The K_{SV}^{app} values depend not only on the quencher efficiency but also on its partition coefficient (K_p) between the aqueous and the lipid phases, and this dependence is described by the equation [46]:

$$\frac{1}{K_{SV}^{app}} = \frac{1}{K_{SV} \cdot K_p} + \frac{1}{K_{SV}} \cdot V_m \qquad (4)$$

Plotting the left hand term against V_m (i.e. a range of lipid concentrations) allows K_{sv} to be determined from the slope and K_p from the intercept (Table **1**).

As observed in Table **1**, the K_p values obtained by both techniques are much higher for tenoxicam and piroxicam than for meloxicam and lornoxicam.

Location Studies by Fluorescence Quenching

In practice, the quenching efficiency of the oxicams was evaluated by addition of increasing amounts of drug to liposome suspensions with incorporated *n*-AS probes. As a result, the fluorescence decreased without causing significant changes in the probes spectral shape (data not shown) and that demonstrates that quenching of *n*-AS probes by oxicams occurs by a collisional mechanism [48]. Fig. **3** is an example of the decrease in fluorescence intensity of *n*-AS probes in EPC unilamellar liposomes (500 μM, pH 7.4) by increasing concentrations of lornoxicam.

Considering this quenching process as collisional, the change in fluorescence is related to the concentration of quencher by equation 3 which can be plotted as a linear function of I_0/I versus $[Q]_T$. The slopes of the Stern-Volmer plots correspond to the values of apparent Stern-Volmer constant, K_{sv}^{app}, for oxicams, which are included in Table **2**.

The use of this set of probes to determine drug location in the bilayer is based on the assumption that all probes have the same intrinsic quenching efficiency [44]. This is true in homogeneous solvents, but in phospholipid bilayers the existence of fluidity and gradient through the plane of membrane is evident. Consequently, it is advisable to calculate bimolecular quenching constant, K_q (Table **2**), which reflects the accessibility of the quencher to the fluorophore and eliminates the microenvironment differences surrounding the probes. This parameter can be determined using the value of
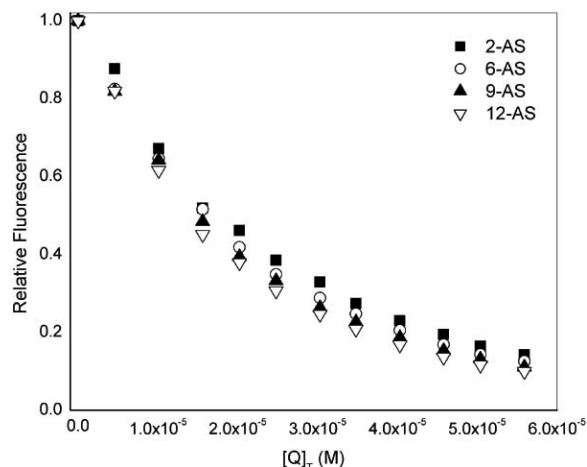

Fig. (3). Fluorescence intensities quenching of *n*-AS probes in EPC unilamellar liposomes (500 μM, pH 7.4) by lornoxicam. Similar plots were obtained for the others oxicams.

excited-state lifetimes (τ_0) found for each probe at pH 7.4 [27, 38, 49-53] in the equation 5:

$$K_q = K_{SV}/\tau_0 \qquad (5)$$

Membrane Fluidity Studies by Fluorescence Anisotropy Measurements

Steady-state fluorescence anisotropy measurements have been widely used to study the influence of several drugs in membrane fluidity and the results are often analysed according to the Perrin equation which relates measured anisotropy to the rotational relaxation time (θ) of the fluorophore; its the fluorescence lifetime (τ), and the fundamental anisotropy of the fluorescent molecule (r_0) [24]:

$$r = \frac{r_0}{1 + (\tau/\theta)} \qquad (6)$$

However, these simple data treatments are incorrect, once that Perrin equation applies only to isotropic rotation of a fluorophore and is not applicable to the anisotropic rotation of the probes in lipid bilayers [24]. Therefore, fluorescent probes can provide reliable qualitative data on overall changes in bilayer fluidity, but it is advisable some caution in literally interpreting data obtained, since that changes in fluidity due to perturbers are not uniform at all segments of the acyl chain and that the probe itself strongly influences the data [24]. In order to provide a sound basis of unambiguous interpretations of the fluorescence probe experiments it is necessary to consider appropriate corrections to experimental data. Complete details of the theory of steady-state anisotropy and its corrections have been described elsewhere [54].

The major problem in the study of membrane fluidity by steady-state fluorescence anisotropy is the existence of hindered rotational motions of fluorescent probes in membranes. Indeed, the anisotropic environment of the membrane hinders the rotation of the fluorophore, the anisotropy decays to a finite value and the depolarization is no longer described by the Perrin equation [24]. Nevertheless, previous studies in membranes [55] have shown that under certain conditions the "out of plane" motion of the *n*-AS probes in membranes

Table 2.   **Values of Apparent Stern-Volmer Constant, K_{sv}^{app}, and the Bimolecular Quenching Rate Constant in the Membrane, K_q, Obtained for Oxicams in Egg Yolk Phosphatidylcholine Unilamellar Liposomes (500 μM, pH 7.4) Labelled with *n*-AS Probes**

Oxicams	K_{sv}^{app}				$K_q \times 10^8 (M^{-1}s^{-1})$			
	2-AS	**6-AS**	**9-AS**	**12-AS**	**2-AS**	**6-AS**	**9-AS**	**12-AS**
Meloxicam	37395±1271	39050±1258	41136±1413	42706±1499	116±3.95	112±3.60	102±3.49	80±2.81
Lornoxicam	114423±2099	122412±2530	149140±2879	167062±3590	468±8.59	461±9.53	485±9.36	413±8.87
Tenoxicam	30829±646	31403±595	31463±993	33426±797	48±1.8	44±0.1	36±0.7	30±0.9
Piroxicam	10504±1258	10601±1055	11327±232	11759±506	15±1.5	14±1.5	13±0.2	10±0.6

[a] The reported values are the mean of at least three independent measurements; the error that affects each value is the standard deviation.

is totally unhindered, in liquid-crystalline phases and whatever the membrane depth. Thus to quantify the effect of NSAIDs in the fluidity of EPC liposomes by steady-state anisotropy measurements using the unmodified Perrin equation, it was necessary to choose the correct experimental conditions. For *n*-AS probes an excitation wavelength of 381 nm must be used [55], and the measurements were performed at 25° C, since EPC vesicles are in a liquid-crystalline phase at this temperature [56].

Besides the limitation to probe rotation existent in an anisotropic media, there is also the problem of the data being influenced by the probe itself. Indeed, there is always the possibility that observed changes in anisotropy, may be caused not only by changes in membrane microviscosity, but also by changes in the excited-state lifetime of the fluorophore, τ' [24]. In the case of the present study, as the quenching between the fluorophore and the quencher is collisional, the decrease in fluorescence intensity is related to that in lifetime [24]:

$$\frac{I_0}{I} = \frac{\tau_0}{\tau'} \quad (7)$$

where I_0/τ_0 and I/τ' are, respectively, the fluorescence intensity/lifetime of the fluorophores in the absence and presence of the quencher. As τ_0 is characteristic of each probe [55] and I_0/I can be obtained by quenching studies, it is possible to calculate τ'.

In summary, using well defined experimental conditions for which one can apply the Perrin equation, the values of the corrected anisotropy (corrected for changes in τ), r', is given by:

$$r' = \frac{r_0}{1 + (\tau'/\theta)} \quad (8)$$

and division of Eq. (6) by Eq. (8) followed by rearrangement yields:

$$r' = \frac{\theta + \tau_0}{\theta + \tau'} \times r_{ss} \quad (9)$$

Then, considering in equation (9) r_{ss} as the value measured for τ_0 (the value in the absence of quencher) and the published values of θ and τ_0 for *n*-AS probes [55], a curve is generated with the values of the corrected anisotropy, r'. These r' values are the variation of anisotropy that should be obtained due to the lifetime changes of the fluorophore (gives a correction for the influence of the probe itself in membrane microviscosity) and are then compared with the experimental r_{ss} values. From the difference between r_{ss} and r' one can see the real variation of anisotropy caused by the drug and without the illusory effect of the intrinsic variation due to the decrease of probe fluorescence lifetime. Once that r_{ss}-r' decreases with increasing oxicams concentration, then it is concluded that a membrane fluidization happened for all the NSAIDs studied. In Fig. **4** is depicted an example of r' and r_{ss} comparison for 12-AS probe in EPC unilamellar liposomes (500 μM, pH 7.4) with increasing effective concentrations of meloxicam and the resultant r_{ss}-r'.

As only drug partitioned into the membrane (effective concentrations) affects the anisotropy, the concentration of NSAIDs used was that partitioned, $[Q]_m$, which can be obtained from [24]:

$$[Q]_m = \frac{K_p [Q]_T}{K_p \alpha_m + (1 - \alpha_m)} \quad (10)$$

where α_m is the volume fraction of membrane phase ($\alpha_m = V_m/V_T$; V_m and V_T represent the volumes of the membrane and water phases, respectively).

The differences in the potency of the compounds studied to increase membrane fluidity are reflected in their IC$_{25}$ values, which are defined as the concentration in membrane phase ($[Q]_m$ in M) of each compound required to increase the fluidising effect ratio $[((r'-r_{ss})/r'_0) \times 100]$ by 25%. Fig. **5** shows an example of the dependence of fluidising effect ratio of *n*-AS probes in egg yolk phosphatidylcholine (EPC) unilamellar liposomes (500 μM, pH 7.4) with increasing effective concentrations, $[Q]_m$, of meloxicam.
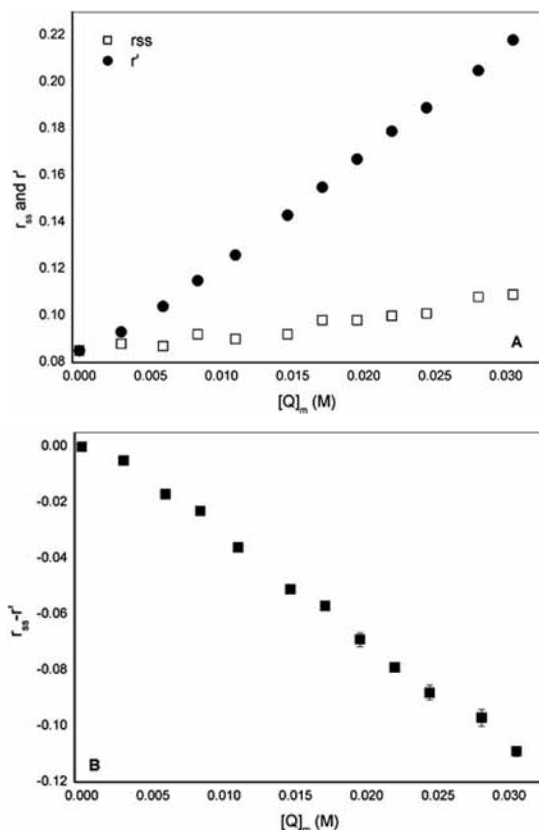
Fig. (4). (**A**) Effect of increasing effective concentrations of meloxicam, $[Q]_m$, in the values of corrected anisotropy, r' and steady-state anisotropy, r_{ss}, for 12-AS probe in egg yolk phosphatidylcholine (EPC) unilamellar liposomes (500 μM, pH 7.4) and resultant r_{ss}-r' (**B**).

The IC_{25} values for each oxicam are given in Table **3** and the order of effectiveness of these compounds in increasing membrane fluidity is lornoxicam> meloxicam > tenoxicam > piroxicam for all *n*-AS probes.

Zeta Potential Measurements

Zeta-potential measurements were made to complete all the information about the binding behaviour of oxicams already gathered by the spectrophotometric and fluorescence techniques. In fact, the interactions of charged drugs with membranes change its surface potential and this can be related to zeta potentials. The determination of zeta potentials of neutral liposomes in the presence of different concentrations of oxicams is represented in Fig. **6**.

There is no significant reduction of the zeta potential values for tenoxicam and piroxicam (the differences were smaller than 3 mV) within the concentration range used; where as for meloxicam and lornoxicam there is a considerable reduction of the zeta potential in a concentration dependent manner.

DISCUSSION

Few studies regarding the physicochemical properties of oxicams were reported and according to them, there are dif-
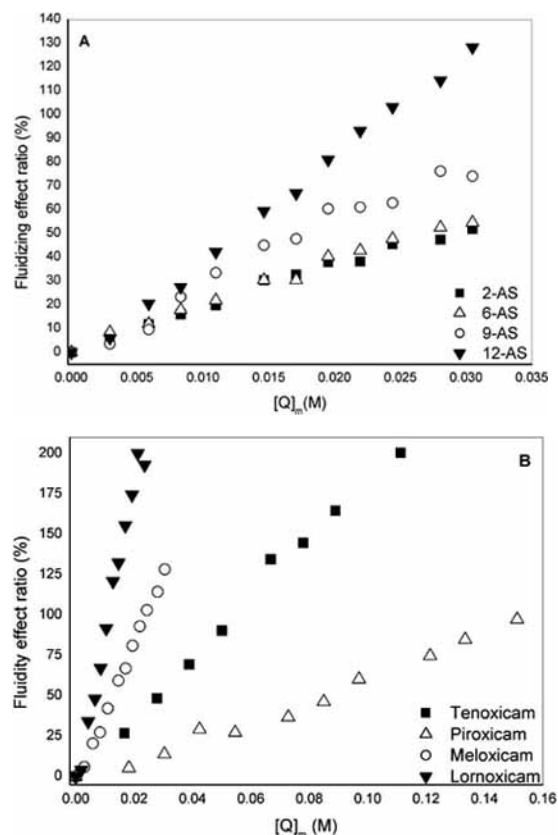
Fig. (5). (**A**) Increase in fluidizing effect ratios of *n*-AS in egg yolk phosphatidylcholine (EPC) unilamellar liposomes (500 μM, pH 7.4) with increasing effective concentrations, $[Q]_m$, of meloxicam.

(**B**) Dependence of the increase in fluidizing effect ratios of 12-AS in egg yolk phosphatidylcholine (EPC) unilamellar liposomes (500 μM, pH 7.4) with increasing effective concentrations, $[Q]_m$, of: tenoxicam, piroxicam, meloxicam and lornoxicam.

ferent ionization forms of these NSAIDs (cationic, neutral/zwitterionic and anionic) [57-62] although there were some contradictory attributions of their ionization constants [63, 64].

The considerations about physicochemical properties of congeneric oxicams and the results obtained in the present study provide evidence that within this NSAID family there is interplay between the ionization forms and the respective behaviour towards the membrane bilayers and regarding this interplay it thus seems possible to set two subgroups inside the oxicam class.

Indeed, the pyridine containing oxicams (tenoxicam and piroxicam) have two opposite charges (positive in pyridine and negative in enol group) in close proximity and are, as a consequence, more stable as zwitterions, even at the physiological pH studied (pH=7.4).

On the other hand, meloxicam, which possesses no pyridine group, is ionized and the dominant specie at the pH studied is the anionic form [61, 62]. Supporting this assumption is the order of Log P_{oct} values (values for partitioning of drugs in octanol/buffer) measured for oxicams at different

Table 3.   Oxicams' IC$_{25}$ Values Determined for Each *n*-AS Probes in Egg Yolk Phosphatidylcholine (EPC) Unilamellar Liposomes (500 μM, pH 7.4)

Oxicams	IC$_{25}$[a] (M)			
	2-AS	**6-AS**	**9-AS**	**12-AS**
Meloxicam	0.0139	0.0131	0.00928	0.00608
Lornoxicam	0.00677	0.00625	0.00459	0.00343
Tenoxicam	0.0327	0.0233	0.0197	0.0138
Piroxicam	0.0840	0.0720	0.0577	0.0410

[a] IC_{25} is the concentration (in M) of each oxicam required to increase the fluidizing effect ratio $[((r' - r_{SS})/r'_0)\times100]$ by 25%.

pH values [61, 62], which shows for meloxicam a significant decrease when the pH is increased from 2 to 7.4 due to deprotonation of enolic OH. The decrease is less marked for tenoxicam and piroxicam due to their predominant zwitterionic population in the aqueous phase in this pH range.

As for meloxicam, the dominant species of lornoxicam at the pH studied are the anionic form. Actually, despite being also a pyridine containing oxicam, lornoxicam has resulted from the replacement of the benzo ring of piroxicam with a chlorothieno ring which may change the electronic properties of neighbouring groups to some extent due to the electron-withdrawing effects of the S-atom [62].
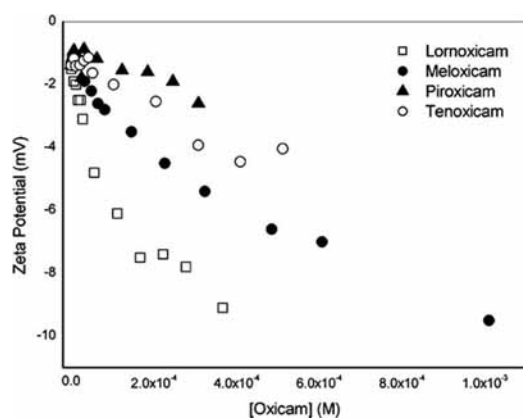
Fig. (6). Dependence of the zeta potential on the concentration of oxicams at pH 7.4 in the presence of 500 μM liposomes.

In conformity with these reported physicochemical differences, tenoxicam and piroxicam which are more stable zwitterions revealed a higher partition capacity in lipid bilayer and consequently higher K_p values, where as meloxicam and lornoxicam, being more negatively charged, had lower partition coefficients (Table **1**). Traditionally, the octanol-water partition coefficient has been used to measure compound hydrophobicity, which was then correlated with drug activity. The octanol-water system is a good membrane model when polar group interactions between the solute and the phospholipid bilayer are minimal or absent, but better systems are needed for molecules which can establish electrostatic interactions with polar groups in the membrane, as

octanol can only model nonpolar interactions [65]. In accordance to this and regardless of the known existence of oxicams' partitioning studies in octanol/buffer systems already mentioned in the literature [61, 62], we have chosen to study their partition in a liposomes/buffer system once that there is a more satisfactory correlation between this parameter and pharmacological aspects for these drugs, especially because they have proved to be able to establish electrostatic interactions with polar groups in the biomembranes. Moreover, liposomes are generally accepted to be a suitable model for the study of membrane structure and properties, given that they are surrounded by a lipid bilayer structurally similar to the lipidic matrix of the cell membranes [25, 29]. Comparing results, one can see that there is no agreement between the partitioning of oxicams in liposomes/buffer and in octanol/buffer systems, however it must be noted that there were also studies of oxicams' partitioning in heptane/buffer which also do not parallel the ones of octanol/buffer systems [62]. In addition, it has been reported that the nature of the microenvironment is capable of modulating the local vicinity of the oxicams leading to a switch over or change between different prototropic forms and selecting a particular form of the drugs for partition (for instance it was referred that the anionic oxicams readily partition into the octanol phase at pH 7.4 and not at all into the heptane phase) [58, 59, 62]. Hence, the differences between the K_p values obtained in this study and the already published Log P_{oct} values could be a matter of different exposed surroundings.

The results of the quenching studies have demonstrated that all the oxicams quench the anthroyloxy group at all membrane depths with relative efficiencies ordered as 12-AS<9-AS<6-AS<2-AS. This slightly decrease towards the internal part of the bilayer suggests that the drugs are located in a similar topography inside the membrane bilayers, independently if they are in a dipole or anionic forms, probably with their hydrophilic part oriented towards the polar part of the membrane bilayers while their hydrophobic segment is in the upper part of the lipophilic tails. This proposed location corroborates other published location studies for oxicams [60]. Referring to lornoxicam's location we can also add that this oxicam reveals a noticeable preference for the 9-AS probe suggesting that a strong additional quenching group (chloride atom) lays near the position 9 of the phospholipids acyl chain.

The data obtained for oxicams' location and partition is also consistent with the zeta potential studies. Analysing the dependence of the concentration of drugs on zeta potential values, Fig. **6**, it is evident that meloxicam and lornoxicam provoke an appreciable decrease of the zeta potential of the bilayer, which confirms that these compounds are negatively charged at the pH of the studies. On the other hand, piroxicam and tenoxicam have less influence over membrane surface potential, which is in agreement with the presence of zwitterionic forms.

The alterations in the membrane fluidity by oxicams mirror their relative quenching efficiencies (which is related to their location within the membrane) once that a perturber preferentially located within the lipid bilayers is more likely to disorder the surrounding lipids, and observing the IC_{25} values (Table **3**) the order of effectiveness of these compounds in increasing membrane fluidity is lornoxicam>meloxicam>tenoxicam>piroxicam.

Besides analysing the differences of the NSAIDs in their ability to perturb the lipid membrane as a whole, it is also important to compare the results obtained for each probe, as they are located in different sites of the membrane and therefore they can report on the microfluidity of those regions. The efficacy of a perturber depends on its general effect on bilayer fluidity and also on its effects at a specific depth of the bilayer. It has been consistently observed for hydrophobic compounds in liquid-crystalline bilayers [28, 52, 66-68], that the greatest perturbation in fluidity occurs in the bilayer centre (closer to 12-AS probe), and the smallest in the plateau region (the gradient is 2 < 6 < 9-AS probes), suggesting that the plateau region is more structurally stable, and less susceptible to perturbation than the bilayer centre. The oxicams studied increase the fluidity and the observed order is 2-AS < 6-AS < 9-AS < 12-AS (Table **3**). This observed order is in agreement with the studies referred above [28, 52, 66-68], as 2, 6 and 9-AS probes, which are part of the plateau region, are less susceptible to perturbation than the 12-AS, located closely to the centre of the bilayer.

CONCLUDING REMARKS

Meloxicam was launched as a novel NSAID of huge therapeutic benefit in the treatment of rheumatoid arthritis, osteoarthritis and other joint diseases possibly due to its reported selectivity for COX-2 [69]. The main question regarding selective COX-2 inhibitors, and why they are better tolerated has led to several proposed theories included chemical structural relationships between meloxicam and other effective NSAIDs with low gastrointestinal toxic effects, namely the presence of sulfa moieties, which determine the preferential binding to COX-2 [69-71]. However, other oxicams have similar chemical groups to those that have been referred to cause COX-2 selectivity, but despite this, are not considered COX-2 selective drugs and exhibit less gastric tolerance.

The studies performed in the current work proved that, in spite of structural similarities, oxicams present different interactions with lipid membranes making possible a virtual division of the class in two groups. Tenoxicam and piroxicam known as COX-1 inhibitors demonstrated higher partition capacity in liposomes/water systems together with a

smaller ability to change the membrane fluidity and surface potential. In contrast lornoxicam and meloxicam, which demonstrated activity against COX-2, have revealed smaller partition capacity in liposomes/water systems together with a higher ability to change the membrane fluidity and surface potential. The found correlations between interactions with membranes; ionization forms and COX inhibition are relevant to understand the pharmacological effects of these NSAIDs. Nevertheless, while meloxicam is a recognised COX-2 selective inhibitor, lornoxicam produces inhibition of COX-2 without a clear selectivity [70-73] and we found no evidence that could support the selective behaviour of meloxicam, although it seems clear that this selective behaviour is not only related with the interaction of this NSAID with biomembranes.

ABBREVIATIONS

COX	=	Cyclooxigenase
EPC	=	Egg yolk phosphatidylcholine
LUVs	=	Large Unilamellar Vesicles
NSAIDs	=	Non steroidal anti-inflammatory drugs
PGs	=	Prostaglandines

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