Tensioactivity and Supramolecular Organization of the Palmitoyl Prodrug of Timolol

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Purpose. To improve the bioavailability of the ocular drug timolol by facilitating its transport through the cornea, an amphiphilic prodrug was synthesized via the addition of a palmitic chain by esterification. The present study was undertaken to investigate the physicochemical and tensioactive properties of the prodrug.

Methods. The amphiphilic properties of the prodrug were firstly investigated by the Wilhelmy plate method. The textures generated by the supramolecular organizations of the ester were visualized by optical microscopy.

Results. The prodrug clearly decreased the surface tension. Optical microscopy provided excellent evidence for the existence of lyotropic liquid crystalline phases: two isotropic but organized phases and a birefringent lamellar phase.

Conclusions. The results from the ensemble of studies undertaken to determine the amphiphilic properties of the prodrug were all in accord with its ability to form liquid crystalline phases. The liquid crystalline state of the prodrug is believed to introduce a delay in the drug pharmacological effect.

KEY WORDS: timolol prodrug; amphiphilicity; surface tension; liquid crystals; microscopy.

INTRODUCTION

Timolol is a β-adrenergic blocker extensively used to decrease intra-ocular pressure in patients suffering from glaucoma. Nevertheless, a number of serious side effects have been reported following its topical ocular administration (1,2). These systemic side effects are believed to be the consequence of poor drug bioavailability in the eye due to (i) precorneal processes that remove the drug rapidly from the absorption site and (ii) the histology of the cornea, which is composed of alternating lipophilic and hydrophilic layers (3). The epithelium and endothelium are more lipophilic than the stroma due to their high concentrations of cellular phospholipids. The stroma itself is largely composed of collagen and water and is hydrophilic (4,5). This reasoning has motivated us to create amphiphilic prodrugs, i.e. having both hydrophilic and lipophilic properties (6), in order to increase bioavailability as some authors have observed it for other prodrugs (7). To this effect, timolol was subjected to esterification by linear aliphatic hydrocarbons ranging from eight to sixteen carbon atoms and was quaternized by the formation of ammonium derivatives after salification with malonic acid. In this study, our attention is focused on the prodrug palmitoyl timolol malonate, which bears the longest aliphatic chain. This choice was motivated by its pharmacological activity (8): only palmitoyl timolol malonate differed significantly from the parent drug timolol maleate in antagonizing isoproterenol-induced ocular hypotension. In addition, prolonged ocular antihypertensive effects were found following the administration of palmitoyl timolol malonate in a betamethasone-induced ocular hypertension model. This could be attributed to the sustained release of timolol from reserves of the prodrug accumulated in the corneal epithelium.

In the following study, we present evidence of this retention due to liquid crystalline phases. Surface tension measurements and optical microscopy were used to characterize this amphiphilic component.

MATERIALS AND METHODS

Chemicals

Levogyre palmitoyl timolol malonate (658 g/mole) was prepared by the esterification of levogyre timolol maleate with palmitoyl chloride (Sigma, Saint Louis, MO, USA). This molecule is represented in Figure 1, with its profile established from NMR results and van der Waals radii (Figure 1a). The details of synthesis have been published elsewhere (6). Palmitic acid was supplied by Prolabo (Paris, France). All other chemicals and solvents were of reagent grade. The following buffer solutions were employed at a ionic strength of 0.1: pH 3.5 (7.6% HCl 0.1M, 92.4% Glycin 0.1M), pH 5 (29.6% CH_3COOH 0.1M, 70.4% CH_3COONa 0.1M), pH 6.5 (13.9% NaOH 0.1M, 50% KH_2PO_4 0.1M, 36.1% KCl 0.1M), pH 8 (46.1% NaOH 0.1M, 50% KH_2PO_4 0.1M, 3.9% KCl 0.1M). Deionized water was used routinely in all preparations (Milli-RO 6+, Millipore, Saint-Quentin-en-Yvelines, France).

Surface Tension Measurements

A tensiometer Lauda Mgw (Konigshofen, Germany) was used and samples were prepared according to the method described by Bangham *et al.* (9) for different prodrug concentrations (0.1, 0.01, 0.001% W/W), in buffer solutions at various pH's (pH 3.5, 5, 6.5, 8). After cleaning of the surface, the tensiometer plate was plunged into the liquid and measurements were recorded over increasing time intervals up to 40 minutes at 22°C. The critical micellar concentration (c.m.c.) was determined in a buffer solution at pH 6.5. Different concentrations were prepared (0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005% W/W) and measurements were recorded until the equilibrium value was attained. About compression isotherms, the Langmuir film balance was a Lauda Fw2 (Konigshofen, Germany). Before spreading monolayers, the air interface of the subphase was cleaned by suction. A chloroform solution of the prodrug was spread onto the buffer solution at pH 6.5 over the maximum available area of 927 cm^2. Monolayers were left for about 15 minutes before measurements were recorded. The rate of compression was 150 cm^2/minute.

Microscopy

A polarizing microscope (Photomicroscope Axiophot Pol, Zeiss, Oberkochen, Germany) was equipped with a quartz first-

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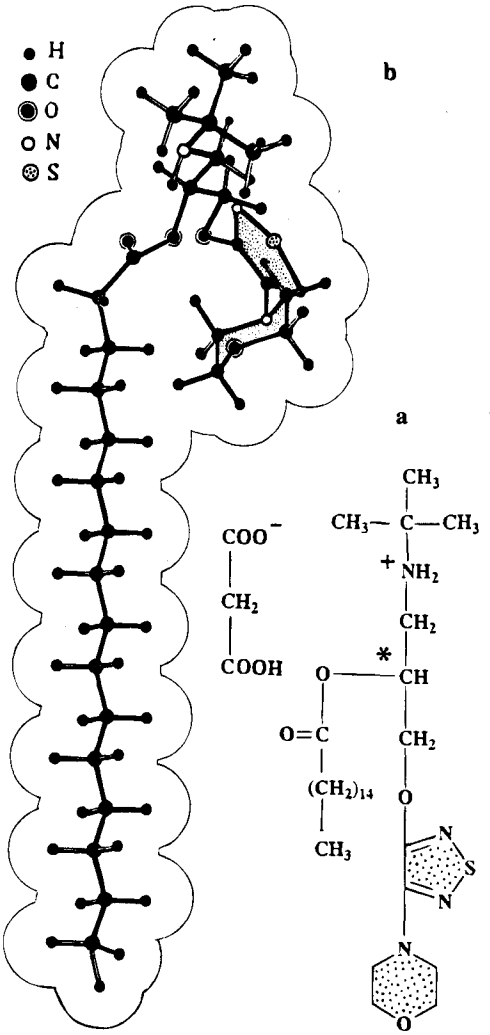

Fig. 1. (a) Chemical structure of palmitoyl timolol malonate with indication of the asymmetrical carbon (*). (b) Three-dimensional view of palmitoyl timolol with a general contour deduced from the van der Waals radii.

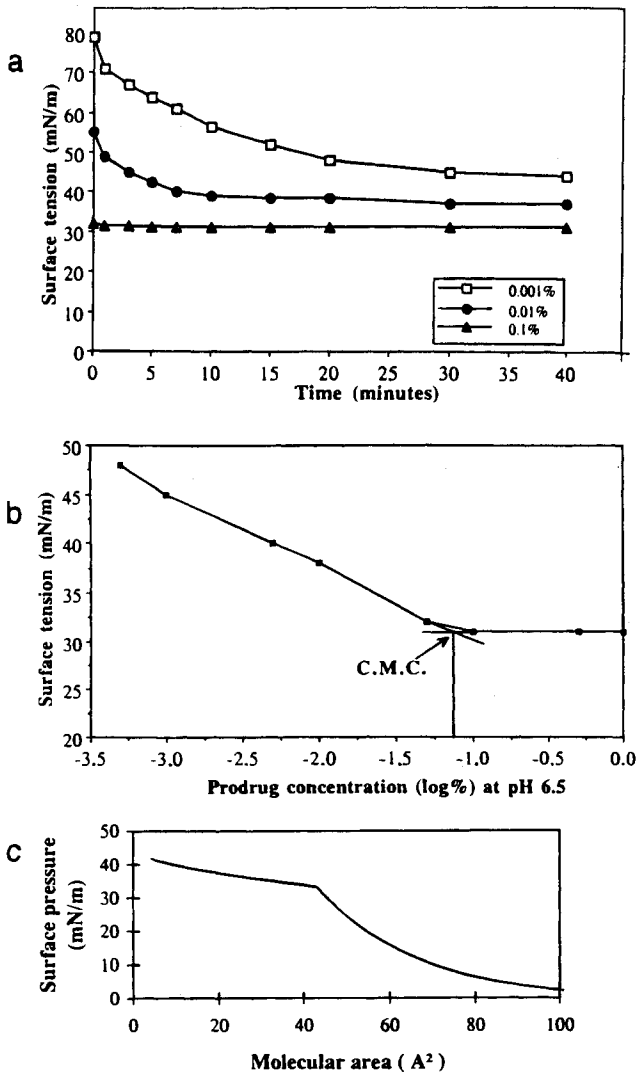

Fig. 2. (a) Decrease of surface tension values versus time and for different concentrations (0.1%, 0.01% and 0.001% at pH 6.5). (b) Determination of the critical micellar concentration of palmitoyl timolol malonate. (c) Compression isotherm of the prodrug monolayer.

order retardation plate and the Nomarski interferential contrast with the corresponding objectives and Wollaston plates. Another polarizing microscope (Orthoplan-Pol, Leitz-Wetzlar, Germany) was equipped for the Zernike phase contrast method. Dispersions of prodrug in water were studied between 90 and 0.6% W/W at ambient temperatures. Owing to the low dispersibility of the prodrug in water, most preparations were briefly heated to 50°C. A thermostated Mettler FP-52 stage (Zürich, Switzerland) was used for microscopic examinations of anhydrous prodrug preparations at temperatures ranging from 20°C to 120°C.

RESULTS

Surface Tension Measurements

The amphiphilicity can be appreciated by measuring the surface tensions of dispersions against time, using the Wilhelmy plate method (10) as illustrated in Figure 2a for pH 6.5.

As expected, the surface tension was lowered as the concentration of the preparation was increased: at pH 6.5, the surface tensions corresponding to concentrations of 0.1%, 0.01%, 0.001% W/W were 31 mN/m, 37 mN/m, 44 mN/m respectively. Kinetic profiles also depend on concentration. For low concentrations (0.001%), at pH 6.5, the initial surface tension value decreased slowly until the equilibrium surface tension value was reached. For higher concentrations (0.1%), the delay to attain the equilibrium value was considerably reduced.

Surface tensions values showed little or no change when the pH was varied in the range of 3.5 to 8. At 0.1%, the following surface tensions were recorded: 27 mN/m at pH 3.5, 28 mN/m at pH 5, 31 mN/m at pH 6.5 and 31 mN/m at pH 8. This lack of influence could be easily understood: at the highest pH (pH 8), the majority of the prodrug's amino groups are very likely protonated. We encountered much difficulty in estimating the pKa of the prodrug, since it is insoluble in aqueous media. However, various prodrugs of timolol which are soluble in

water, synthesized notably by Bundgaard *et al.* (11), have been shown to have pKa's of ca. 8.4. We therefore have good reason to believe that the pKa of palmitoyl timolol malonate is near to this value and argue that an overwhelming proportion of the molecules are in the ionized form at pH 8. The prodrug's surface-active behaviour was naturally quite similar in the pH range studied.

The variation of surface tension values plotted against concentration at pH 6.5 is shown in Figure 2b. After a linear decrease, the surface tension remains constant. The point at the intersection of the two regression lines at 0.07% W/W (logarithm value of −1.15) can be defined as critical micellar concentration (c.m.c.) and corresponds to a molar concentration of 1.06 mM. pH and ionic strength effects on the prodrug c. m. c. will be considered in a further work.

With the Langmuir film balance, we observed that the monolayer was stable with no observable hysteresis. The minimal area per molecule is deduced from the compression isotherm of the prodrug monolayer and corresponds to the abscissa of the inflexion point (Figure 2c). A minimal area of 43 $Å^2$ per molecule was recorded.

Microscopy Observations

In its anhydrous state at room temperature, the prodrug exhibited an amorphous aspect with birefringent microcrystals scattered in all directions. Some of them formed spherulitic shapes with irregular Maltese crosses. At 50–60°C, complete isotropic melting was observed. On cooling this liquid, crystals reappeared forming radiating patterns. After reheating, melting occurred regularly at 47°C.

At room temperature, the dispersion of palmitoyl timolol malonate in water is heterogeneous irrespective of the concentration (Figure 3a), with the coexistence of several phases observed in interferential contrast. Solid crystals (cr) and liquid crystals (L) could be recognized (Figure 3a) along with two different isotropic phases (i_1 and i_2). Small solid crystals persisted, even at concentrations of 20%. The two isotropic phases were present together for concentrations ranging from 0.60% to 90%, what corresponded to an extremely large interval for these two phases in equilibrium.

The isotropic phase i_1 was probably less concentrated than i_2, which contained most of the true prodrug crystals. Phase i_1 also exhibited a smaller refractive index than i_2: this was deduced from phase contrast methods in constant thickness domains. The greater viscosity of phase i_2 was also appreciated by moving the coverslip slightly.

Smectic type liquid crystalline phases, also referred to as 'lamellar phases', were observed in some preparations by the presence of myelinic systems, with characteristic labyrinth-like patterns L (Figure 3a). This phase is mainly observed for concentrations from 7.5% to 1.25%, at interfaces i_1, i_2.

The myelin figures of palmitoyl timolol malonate are well visualized in polarizing microscopy (Figure 3b) and are similar to those developed by amphiphilic molecules such as phospholipids. In Figure 3b, cylindrical and birefringent myelinic tubes are immediately recognized and, when water evaporates, these tubes flatten and bilayers align horizontally, appearing black between crossed polars, whereas sets of bilayers remain vertical or oblique observed as brilliant stripes usually called 'oily streaks'. These black domains separated by bright walls are often named 'cellular textures' (Figure 3c) and are a classical texture of lyotropic lamellar phases.

In phase contrast microscopy, the cylindrical fingers of the myelinic systems show various alterations, narrowings for instance (n), as may be seen from the isolated rodlet in Figure 3d. Myelinic figures and lamellar textures easily deform in fluid streams, visible in the microscope and the resulting shapes are shown in Figure 3d.

DISCUSSION

As expected, the esterification of timolol maleate with a palmitoyl chain and creation of an hydrophilic group (quaternary ammonium salt) afforded an amphiphilic prodrug which could be characterized by surface tension measurements and the spreading of its monolayer on the Langmuir film balance. The critical micellar concentration (c.m.c.) at pH 6.5 was found to be 0.07%. However, this concentration was lower than the concentration employed in the different assays described herein.

The obtained results are characteristic of the tensio-active behaviour observed for other amphiphilic compounds, which are also known to form liquid crystals.

In palmitoyl timolol malonate, three different types of phases are observed: the lamellar phase clearly identified by its textures (myelinic figures, oily streaks, Maltese crosses) for concentrations from 7.5 to 1.25%; two isotropic phases coexisting between 90 and 0.6%.

The interpretation of the different phases comes mainly from the conformational study of the prodrug in deuterochloroform or in deuterated water (6): the study was made by NMR using Nuclear Overhauser Effect Correlation Spectroscopy (NOESY). In a polar medium such as deuterated water, the absence of dipole-dipole relaxations between the different parts of the molecule led us to conclude that the molecule adopted an extended molecular conformation as described in Figure 1b and quite compatible with a supramolecular arrangement in this medium: we observe an unfolding of the paraffinic chain and a spatial distancing between the morpholinic ring and the tertiobutyl group. It is therefore possible to calculate that the surface occupied by the whole polar head (morpholino-thiadiazole moiety) is about 40 $Å^2$ which corroborates the 43 $Å^2$ area obtained with the Langmuir film balance.

The area of the hydrophilic head is nearly twice as that occupied by the fatty chain in cross section and this is a noteworthy point in the interpretation of the different structures. This truncated conic shape leads to the formation of cylindrical micelles (Figure 4a). On the contrary, if the prodrug paraffinic chains interdigitate, the area occupied by two fatty chains is also about 40 $Å^2$ like the polar head and this favours the smectic phase made of superposed bilayers of the prodrug (Figure 4b). The existence of a lamellar phase intercalated between two isotropic phases is frequent in lyotropic liquid crystals and we suppose that this situation is realized in our system. The isotropic phases encountered in such situations are either cubic liquid crystalline or correspond to 'amorphous liquid crystals' also named 'sponges'. The model of Figure 4c associates the structures of these two isotropic 'sponges'. One is constituted of elongated and branched cylindrical micelles (upper part of Figure 4c); the other one is made of a unique bilayer of complex shape, the median bilayer surface being toroidal (lower part of Figure 4c), separating two intertwined aqueous compartments.

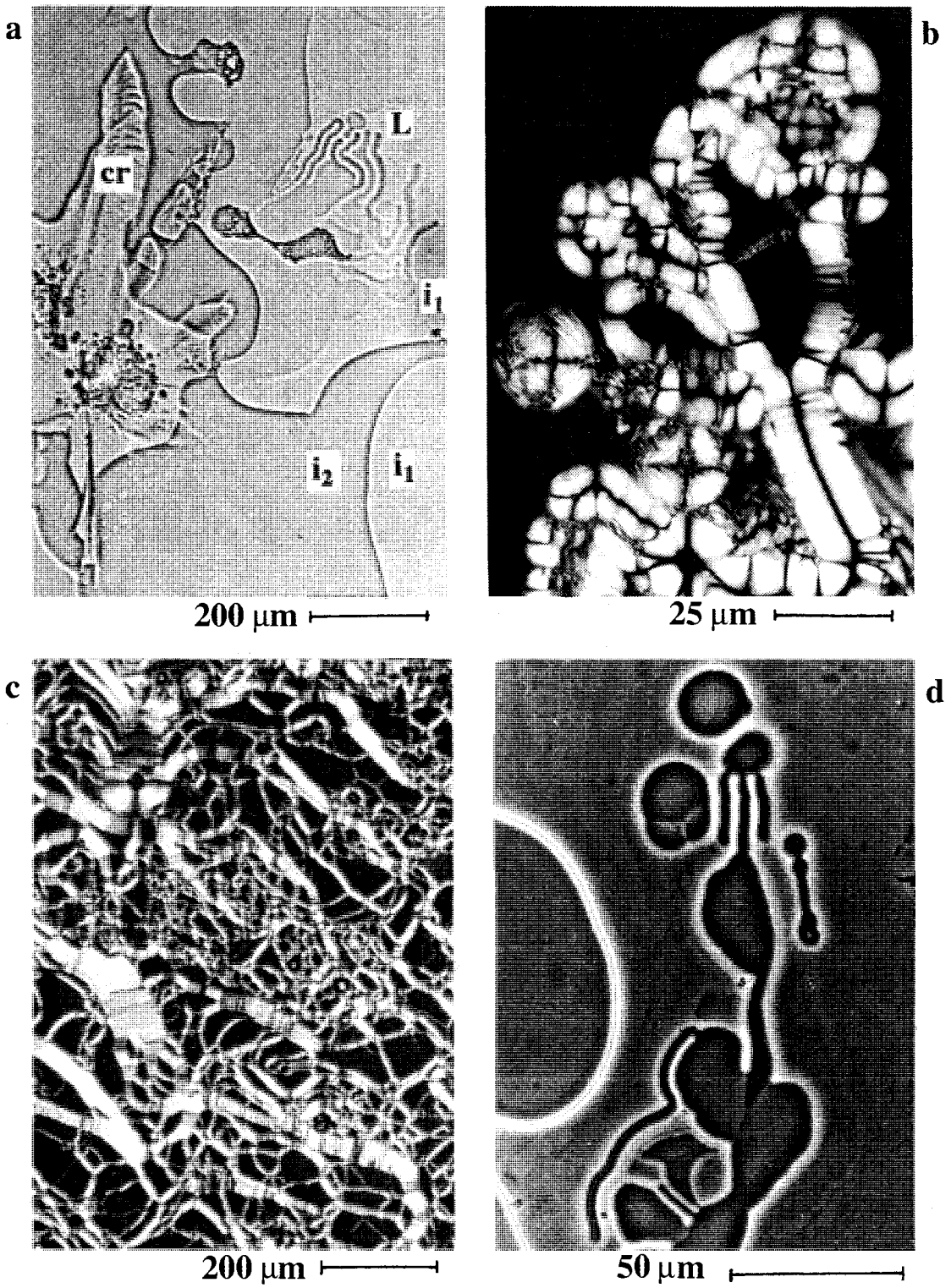

Fig. 3. Microscopy of palmitoyl timolol malonate dispersed in water. (a) Different phases in interferential contrast microscopy. Solid crystals (cr) are present at the top of the micrograph, whereas myelinic systems in the form of "labyrinths" are recognizable at the bottom (L). An interface separates two different isotropic phases, i_1 and i_2, the most refringent one (i_2) being much more viscous (20%). (b) Lamellar phases of palmitoyl timolol malonate observed between crossed polars: well developed myelinic sytems, after a brief heating (20%). (c) A cellular texture formed of oily streaks, with numerous cross striations, obtained after a brief heating (20%). (d) Myelinic tubes can form separate rodlets and often show characteristic narrowings (n) (2.5%).

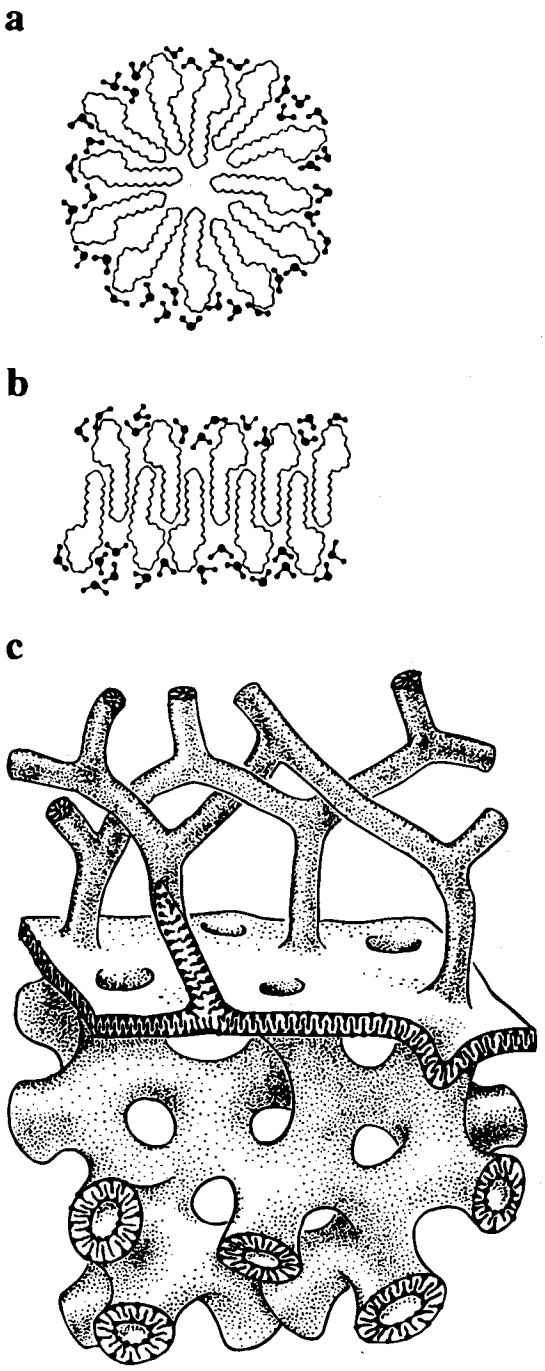

Fig. 4. (a) Arrangement of palmitoyl timolol malonate in a cross section of a cylindrical micelle. Some water molecules are represented at the periphery, facilitating the fan distribution of molecules. (b) Cross-section of a bilayer, with interdigitating palmitoyl chains. (c) Three-dimensional model of the interface separating the two 'sponges', the upper one, with branching cylindrical micelles being much more water rich than the lower one, with a toroidal bilayer separating two water compartments.

This is hypothetic, but nevertheless plausible in the light of recent work (12–14).

If demonstrated, the structure of these phases may explain the significant differences in the prodrug's in vivo activity when compared with the reference molecule, timolol maleate. In a betamethasone ocular hypertension model, the decreased intraocular pressure produced by the palmitoyl timolol malonate at a concentration of 1% W/W was more pronounced at longer times (8).

The lamellar phase is analogous to the organization of physiological membranes such as the corneal epithelium. This facilitates the absorption of the prodrug within epithelium cell membranes. However, since the prodrug assembles into liquid crystals, its mean residence time in the corneal tissues can be dramatically enhanced, supporting a sustained delivery. It is worth remembering that timolol has been associated to a cubic phase of a surfactant, the Brij®96 (POE-oleic alcohol ether) and that the ocular release of the drug is slowed down (15). The isotropic phases of the prodrug proposed by this study could produce a similar phenomenon, since their probable sponge-like structures are very close to cubic phases.

CONCLUSIONS

The above physico-chemical study, including surface tension measurements and optical microscopy, has provided convincing evidence for the amphiphilic character of the palmitoyl timolol malonate and its capacity to form liquid crystalline phases (two isotropic phases and a smectic phase). These properties must be taken into account when considering the pharmacological activity of this prodrug. Better diffusion across the epithelium can be achieved by the attachment of a paraffinic chain to the drug. Likewise, its ability to assemble into supramolecular systems ensures the drug's protection against rapid side diffusion.

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