ELSEVIER

International Journal of Pharmaceutics 128 (1996) 179–188

international journal of pharmaceutics

A timolol prodrug for improved ocular delivery: synthesis, conformational study and hydrolysis of palmitoyl timolol malonate

Brigitte Pech[a], Olivier Duval[b], Pascal Richomme[c], Jean-Pierre Benoit[a,*]

[a]*Laboratoire de Pharmacie Galénique et de Biophysique Pharmaceutique, Faculté de Pharmacie. Université d'Angers, 16, Boulevard Daviers, 49100 Angers, France*

[b]*Laboratoire de Chimie Thérapeutique, Faculté de Pharmacie. Université d'Angers, 16, Boulevard Daviers, 49100 Angers, France*

[c]*Service de RMN, Faculté de Pharmacie. Université d'Angers, 16, Boulevard Daviers, 49100 Angers, France*

Received 5 June 1995; accepted 25 July 1995

Abstract

The synthesis of prodrugs exhibiting amphiphilic properties could prove to be fruitful in overcoming systemic effects and improving the intraocular bioavailability of timolol administered topically in the eye. In this study, an amphiphilic prodrug of timolol, palmitoyl timolol malonate, was synthesized by the esterification of timolol with a palmitoyl chain and its structure was characterized by spectroscopic analysis. NMR studies were performed using nuclear Overhauser effect correlation spectroscopy (NOESY) in two deuteriated solvents (D_2O and $CDCl_3$) to determine the conformation of palmitoyl timolol malonate. In deuteriated water, dipole-dipole relaxations between the different parts of the molecule did not appear. The absence of such relaxations, implying an extended molecular conformation, underlines the amphiphilicity of this prodrug and points to a supramolecular arrangement in water. The relative stability of the ester prodrug was borne out by its hydrolysis in various aqueous solutions: its stability was found to increase as the pH and the ionic strength of the medium increased. An increase in stability was also noted as the concentration of the ester was decreased. Assays in the presence of corneal homogenates showed that the hydrolysis was essentially mediated by esterases.

Keywords: Timolol prodrug; Palmitoyl timolol malonate; Synthesis; Conformation study; Nuclear magnetic resonance; Chemical hydrolysis; Corneal homogenates mediated hydrolysis

1. Introduction

β-Adrenergic blockers were first reported to be useful in the treatment of glaucoma (or ocular hypertension) in 1967. Their pharmacological action might be accredited to a reduction of the secretion of the aqueous humor at the ciliary process levels.

In 1978, timolol ((*S*)-(−)-1-[tert-butylamino]-3-[(4-morpholino-1,2,5-thiadiazol-3-yl)oxy]-2-propanol) maleate was approved for market use, and has since become very popular with opthal-

* Corresponding author. Tel.: 41 481422; Fax: 41 486733.

SSDI 0378-5133(95)04275-F

mologists as an effective antiglaucoma agent. However, a vast number of serious cardiovascular, respiratory, central nervous system and ocular side effects, secondary to topical ocular timolol administration, have been reported (Nelson et al., 1986). In fact it has been estimated that only 1–2% (or less) of an instilled dose penetrates the inner eye, the remaining dose reaching the systemic circulation. This poor availability is largely due to precorneal processes that remove the drug rapidly from the absorption site, and to the lipophilicity of the corneal epithelium which restricts corneal drug penetration. Thus far, potential approaches to decrease the systemic absorption of topically applied timolol, thereby diminishing its adverse effects, have involved the use of prodrugs with high affinities for the cornea. In most studies, the aim is to synthesize prodrugs with increased lipophilicities so that the absorption through the corneal lipophilic epithelium is improved.

In adopting such an approach, it is worthwhile considering the structure of the cornea as a whole. In practical terms, if the epithelium and the endothelium are lipophilic in nature and if the stroma is very hydrophilic, then increasing prodrug lipophilicity could be disadvantageous for ocular absorption.

These reasons have motivated us to create amphiphilic prodrugs, i.e., having both hydrophilic and lipophilic properties. In a recent publication (Pech et al., 1993), a series of amphiphilic esters of timolol malonate (octanoyl, decanoyl, dodecanoyl, myristoyl and palmitoyl timolol malonates) were tested in rabbits, for their capacity to antagonize the isoproterenol-induced ocular hypertension, using timolol maleate as the reference drug. The most active compound, the ester with the longest aliphatic chain (palmitoyl timolol malonate), showed its ability to decrease intraocular pressure in a betamethasone-induced ocular hypertension model, at the longest experimental times (4, 5 and 6 h after administration), when compared to timolol maleate.

In this study, we report the synthesis of palmitoyl timolol malonate and its conformation as a function of solvent polarity, as determined by NMR techniques. The chemical stability of the ester was evaluated by its dissolution in different aqueous solutions. The enzymatic hydrolysis of the amphiphilic prodrug in corneal homogenates was also studied.

2. Materials and methods

2.1. Apparatus

^{1}H-NMR spectra were recorded on a Jeol GSX 270 WB apparatus (Tokyo, Japan). Positive-CI mass spectroscopic analyses were conducted on a Nermag R1010 C mass spectrometer (Argenteuil, France). Infrared spectra were performed on a Perkin-Elmer 580 spectrometer (Palo Alto, CA, USA). Optical rotations were measured on a Schmidt-Haensch polarimeter (Berlin, Germany). HPLC was performed with a Waters apparatus (Pump M 45, automated gradient controller and photodiode array detector 991) (Milford, USA).

2.2. Chemicals

Levogyre timolol maleate and palmitoyl chloride were purchased from Sigma Chemicals (St. Louis, MO, USA). Deuterochloroform and deuteriated water were obtained from Merck (Darmstadt, Germany). Buffer substances and all other chemicals or solvents were of reagent grade.

2.3. Synthesis of palmitoyl timolol malonate

Timolol maleate and palmitoyl chloride were used in stoichiometric quantities (Fig. 1). Triethylamine (0.48 ml, 3.45 mmol), dried over lithium-aluminium hydride and freshly distilled, was added to a solution of timolol maleate (1 g, 2.3 mmol) in acetonitrile at 0°C. Palmitoyl chloride (0.63 g, 2.3 mmol) was then introduced and the mixture stirred at 80°C for 24 h. Thereafter, triethylamine was added (3.45 mmol). After standing for one day at 80°C, the solvent was evaporated under reduced pressure and the residue was taken up in ethyl acetate (100 ml) and washed with water (2 × 200 ml), 0.1 N HCl (200 ml), 0.1 N NaOH (200 ml) and water (200 ml), respectively. The organic layer was dried over

anhydrous sodium sulphate and evaporated under vacuum to give the timolol ester-free base. The quaternary ammonium salt was obtained following the addition of malonic acid: after dissolution in diethylic ether (60 ml), a solution of malonic acid (2.3 mmol) in 2-propanol (4 ml) was added. After evaporation of the solvents under reduced pressure, palmitoyl timolol malonate was isolated as a viscous mass (0.33 g, 0.506 mmol).

Two methods were used to determine the purities of the prodrugs.

2.3.1. High performance liquid chromatography

After dissolution in chloroform, the analysis of the different bases synthesized was performed using a stationary phase Lichrospher Merck 100 CN (4 × 250 mm) (Darmstadt, Germany). The mobile phase was chloroform (2.5 ml/min) and detection was monitored at 294 nm.

2.3.2. Nuclear magnetic resonance

^{1}H-NMR spectroscopy was employed to determine product purities after dissolution of the prodrug in deuterochloroform. A comparison between the integration curves of signals corresponding to the tertiobutylic group of timolol (singlet of nine protons at δ 1.15 ppm) and the terminal methyl group of the aliphatic chain (triplet of three protons at δ 0.87 ppm) allowed us to verify the degree of esterification of the prodrug and/or the amount of residual acid chloride in the preparation. Washing with petroleum ether or extraction in acidic or basic conditions was then repeated until the desired ratio of proton integrations was obtained.

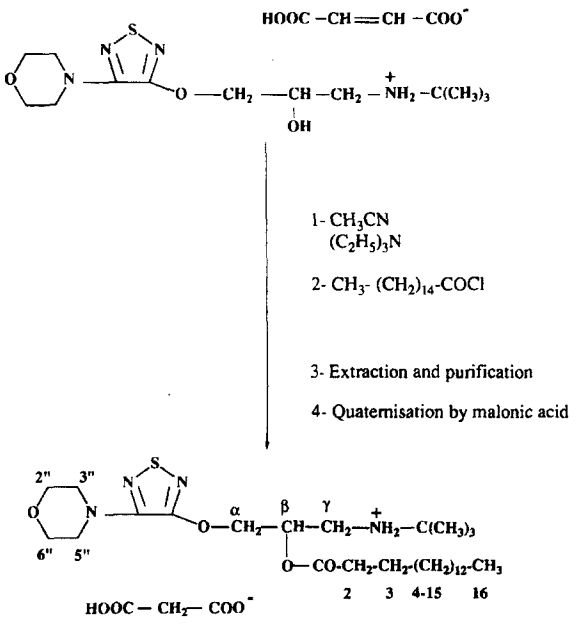

Fig. 1. Synthesis of palmitoyl timolol malonate.

2.4. Conformational study

A deuteriated polar solvent (D_2O) and an apolar solvent ($CDCl_3$) were chosen for the conformational studies. For the prodrug in deuterochloroform, samples were prepared by simple dissolution followed by filtration. For samples in deuteriated water, dispersions were made according to the method proposed by Bangham et al. (1965) and used for the preparation of liposomes: the prodrug was dissolved in chloroform in a round-bottomed flask and the solvent was evaporated at 50°C under reduced pressure. The film formed on the surface of the flask was then dispersed in deuteriated water under continuous stirring.

2.5. Hydrolysis in aqueous solutions

The hydrolysis of the palmitoyl timolol malonate was studied at 37°C at two different concentrations (3×10^{-4} and 7.9×10^{-3} M/l), at a ionic strength of $\mu = 0.1$ and at different pH values: 3.5 (7.6% HCl 0.1 M, 92.4% glycine 0.1 M), 6.5 (13.9% NaOH 0.1 M, 50% KH_2PO_4 0.1 M, 36.1% KCl 0.1 M), 7.5 (40.9% NaOH 0.1 M, 50% KH_2PO_4 0.1 M, 9.1% KCl 0.1 M), 9.5 (73.2% glycine 0.1 M, 26.8% NaOH 0.1 M). An ionic strength of $\mu = 0.5$ was also used with buffer solutions of 0.5 M.

Samples were prepared according to the method of Bangham et al. (1965) as described above.

At designated time intervals, samples were taken and diluted with the same quantity of methanol. After vigorous shaking, samples were assayed immediately by HPLC. The rates of hy-

drolysis were determined using a reversed-phase HPLC procedure, capable of separating the ester from timolol. The column used was a C18 column (3.9 × 300 mm, μ-Bondapack®, Waters, Milford, USA). The mobile phase was composed of a solution of 40% v/v acetonitrile in 0.06% orthophosphoric acid, while the flow rate was 2 ml/min. The column effluent was monitored at 294 nm and the rate of hydrolysis of the prodrug was determined by appearance of free timolol.

2.6. Hydrolysis in corneal homogenates

The eyes of two newly killed male New-Zealand albino rabbits (Charles River France, Saint Aubin lès Elbœuf, France) were rapidly removed and placed in an ice-cold isotonic potassium chloride solution. Immediately following their removal, corneas were excised from each eye using a surgical scalpel. The four corneas were transferred to a homogenization vessel and homogenized with 3 ml of an ice-cold isotonic potassium chloride solution in a Potter-Elvejhem tissue homogenizer (Bioblock, Illkirch, France). The homogenate was centrifuged at 2000 rpm in a refrigerate centrifuge at 4°C (SR 2022, Jouan, Paris, France) for 5 min and the supernatant was isolated for enzymatic hydrolysis.

Thereafter, 700 μl of corneal supernatant was added to the prodrug (8.27×10^{-4} mmol) in 5 ml of Tris buffer at pH 7.4 (prepared according to the method of Bangham et al. (1965)). The final concentration was 1.45×10^{-4} M/l.

Chromatographic analyses were conducted according to the method described in the assay procedure developed for chemical hydrolysis.

3. Results and discussion

3.1. Synthesis and structural analysis

Palmitoyl timolol malonate (658 g/mol) was obtained in a yield of 22% and was structurally characterized using a combination of analytical techniques:

infrared spectroscopy (KBr): 2900 cm^{-1} (aliphatic chain), 1750 cm^{-1} (C = 0 ester);

optical rotation ($CHCl_3$, c = 1): $[\alpha]_D = -20°$;

mass spectrometry (NH_3) (base): 555 $[M + 1]^+$ (100%), 368 (21.2%), 112 (39.1%);

^{1}H-NMR (270 MHz, $CDCl_3$, TMS = 0, δ ppm): 0.87 (t, 3H, J = 8.3 Hz, H_3-16); 1.24 (s, 24H, H_2-4 to H_2-15); 1.40 (s, 9H, CH_3 t-butyl); 1.57 (m, H_2-3); 2.33 (m, H_2-2); 3.11 (s, CH_2 malonate); 3.14 (m, 1H, H′-γ); 3.43 (d, 1H, J = 17.3 Hz, H″-γ); 3.47 (m, 4H, H_2-3″ and H_2-5″); 3.79 (m, 4H, H_2-2″ and H_2-6″); 4.62 (dd, 1H, J_{gem} = 13.0 Hz and J_{vic} = 9.0 Hz, H′-α); 4.67 (dd, 1H, J_{gem} = 13.0 Hz, J_{vic} = 9.0 Hz, H″-α); 5.48 (m, 1H, H-β).

^{13}C-NMR (67.5 MHz, $CDCl_3$, TMS = 0, δ ppm): 14.1 (C-16); 22.7 (C-15); 24.7 (C-3); 25.8 (CH_3 t-butyl); 29.1 to 29.7 (C-4 to C-13); 31.9 (C-14); 34.0 (C-2); 38.7 (CH_2 malonate); 42.5 (C-γ); 47.8 (C-3″ and C-5″); 57.2 (C-t-butyl); 66.4 (C-2″ and C-6″); 68.6 (C-α); 69.2 (C-β); 149.6 (C-3′); 152.9 (C-4′); 173.5 (C-1).

Assignments of proton and the carbon resonances of the molecule were completed through ^{1}H-^{1}H COSY and ^{1}H-^{13}C COSY experiments (HETCOR spectrum in Fig. 2).

3.2. Conformational study

The influence of the polarity of the solvent on the conformation of the prodrug was determined by ^{1}H-NMR spectroscopy (T1 measurements and nuclear Overhauser exchange spectroscopy (NOESY)).

In nOe spectroscopy (nuclear Overhauser effect), a change of intensity of one resonance is measured when the transitions of others are perturbed. If two spins are separated by a distance r, the nOe will be proportional to r^{-6}. NOESY was developed to observe nOe two-dimensionally (peaks off the diagonal are cross peaks due to proton pairs showing Overhauser effects (i.e., spatially close protons)).

Thus, nOes between all protons can be observed simultaneously and since nOe contains important information for the determination of inter-proton distances, it is a useful experimental technique in molecular structural analysis.

In $CDCl_3$, the multitude of cross peaks (Fig. 3) clearly indicated dipole-dipole relaxations between

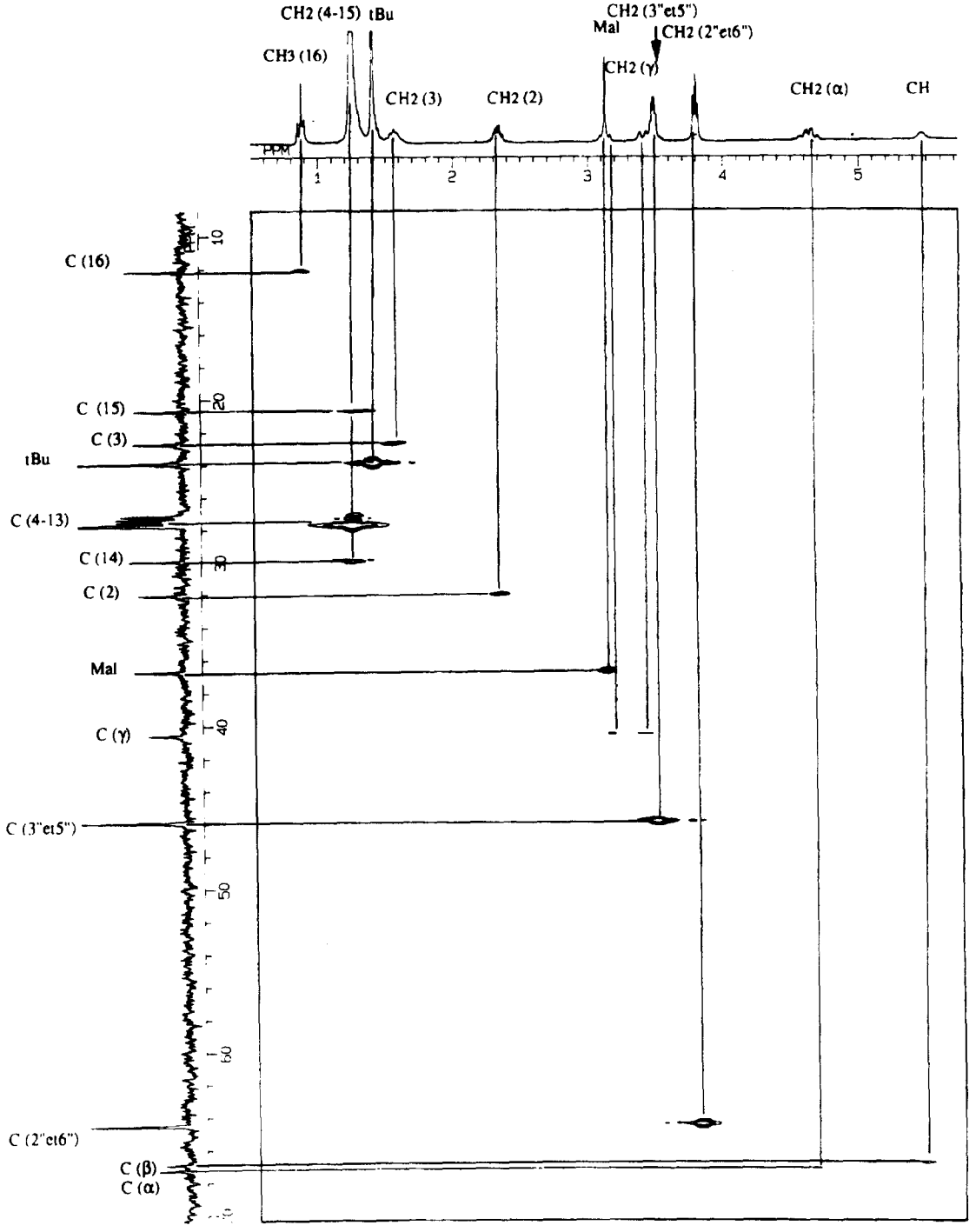

Fig. 2. HETCOR spectrum of palmitoyl timolol malonate.

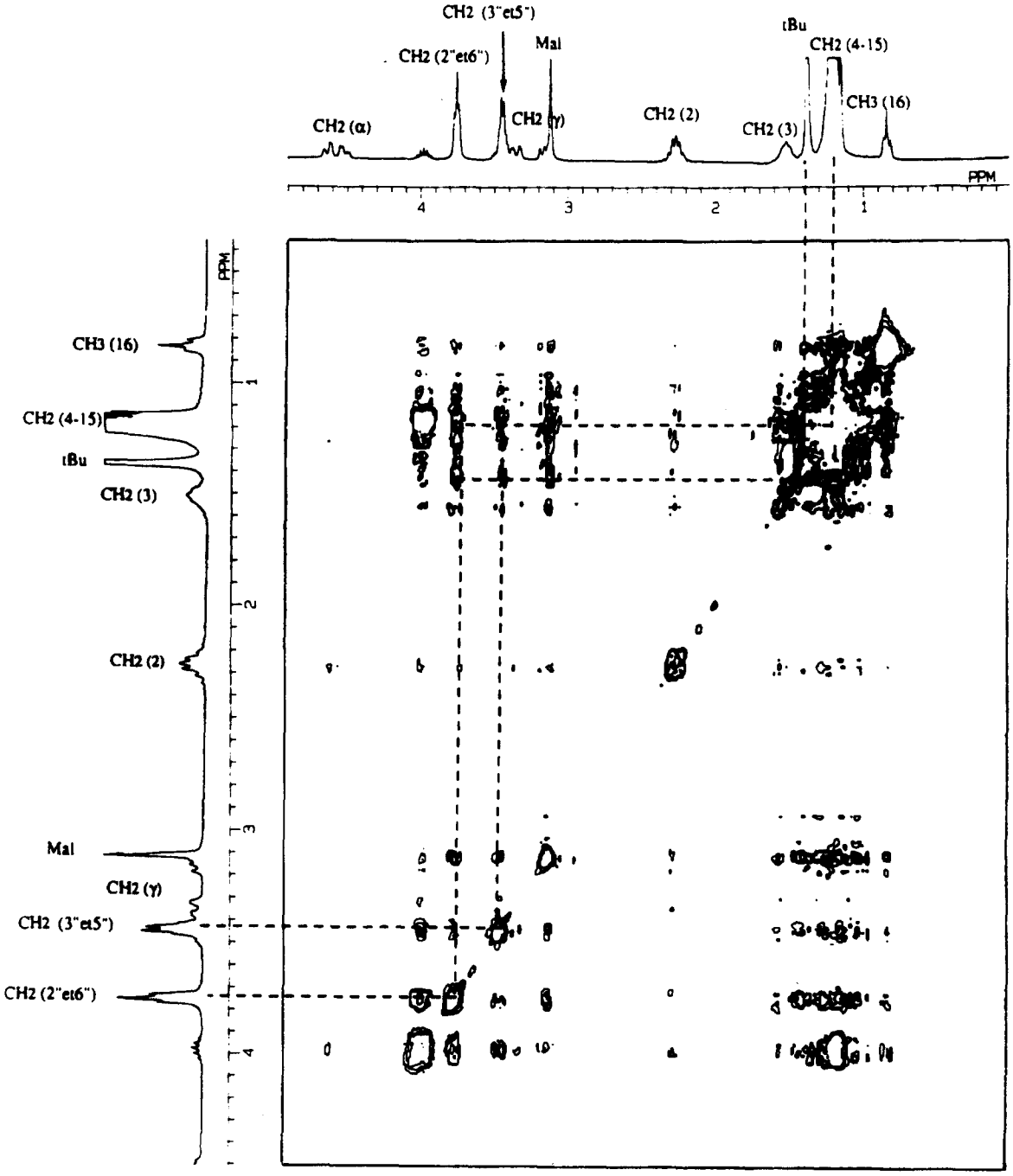

Fig. 3. NOESY ($CDCl_3$) spectrum of palmitoyl timolol malonate.

the aliphatic and the morpholinic protons in the ester prodrug. This provided strong evidence for the wrapping of the hydrocarbon chain around its morpholino-thiadiazole centre of anchorage. Interactions were also observed between the ter-butylic protons and those of the morpholinic group.

In deuteriated water, nOe interactions were insignificant, or did not appear, indicating the spatial distancing of the morpholinic ring and the tertiobutyl group. The polarity of the solvent thus appears to induce an extension of the oleofinic chain (Fig. 4). This fact supports the amphiphilicity of the palmitoyl timolol malonate and suggests that prodrug molecules adopt supramolecular arrangements in water.

3.3. Hydrolysis kinetics in aqueous solutions

Within the pH range investigated (pH 3.5–9.5), palmitoyl timolol malonate was found to be quantitatively hydrolysed to timolol. As indicated by HPLC, the disappearance of ester was accompanied by the concomitant appearance of free timolol. Photodiode array detection (190–800 nm) demonstrated that no peaks, other than those corresponding to timolol, appeared during the hydrolysis of the ester. This fact shows that there was no intra-molecular aminolysis of the ester to give the corresponding *N*-acyl timolol derivative. Aminolysis has been observed with esters of various β-blockers including propranolol (Buur et al., 1988) and oxprenolol (Jordan et al., 1992). The inability of palmitoyl timolol malonate to undergo intramolecular aminolysis may be due to the steric hindrance exhibited by the bulky tertiary butylamino group.

It must be pointed out that an amphiphilic prodrug like palmitoyl timolol malonate does not form true solutions in water. Above the critical micellar concentration, it forms micelles or other liquid crystalline structures (Pech et al., 1995). Consequently, the hydrolysis of supramolecularly organized prodrug molecules in buffer solutions did not follow the classical first-order kinetics found for esters in solution.

The influence of pH, ionic strength and prodrug concentration on the analysis of hydrolysis products is shown in Table 1. The rate of hydrolysis was based on the time taken (in days) for the prodrug concentration to decrease to half that of its original value. An increase in the stability was observed as the pH of the medium was increased: the maximal stability being found at pH 9.5. For the same ionic strength (μ = 0.1) at pH 6.5 and for a higher concentration (7.9×10^{-3} instead of 3×10^{-4} M/l), the stability decreased. At pH 6.5 and for a concentration of 3×10^{-4} M/l, if the ionic strength was increased (μ = 0.5), the stability of the prodrug was notably increased.

Irrespective of the experimental conditions, we observed a notable increase in stability in comparison with esters of timolol synthesized by Bundgaard et al. (1986) (acetic ester, $t_{1/2}$ = 28 min; propionic ester, $t_{1/2}$ = 40 min; butyric ester, $t_{1/2}$ = 50 min; and pivalic ester, $t_{1/2}$ = 215 min, for 0.1 mM of ester in 0.02 M phosphate buffer at pH 7.4 and at 37°C). This increase in stability may be related to the increase of the chain length, a theory forwarded by several authors. Tojo et al. (1988) noted that the rate of hydrolysis of cataline prodrugs is maximal for a two-carbon atom chain, whereas it decreases for longer chains. Maksay et al. (1980) pointed out that increased stabilities of some esters of benzodiazepines may be induced by an increasing number of carbon atoms on side-chains. We believe that the same phenomenon occurs in the case of palmitoyl timolol malonate, although it cannot be ruled out that the increased stability may be due to the formation of prodrug micelles, or other liquid crystalline phases, as a consequence of their poor solubility in aqueous media.

The increased stability with increasing ionic strengths at lower pHs is more consistent with an intramolecular general acid catalysed hydrolysis which involves ions of opposite charges (mechanism a in Fig. 5) than the less likely intramolecular general base catalysis (mechanism b in Fig. 5).

3.4. Hydrolysis in corneal homogenates

Fig. 6 gives the percentage of prodrug remaining after hydrolysis with respect to time. Enzy-

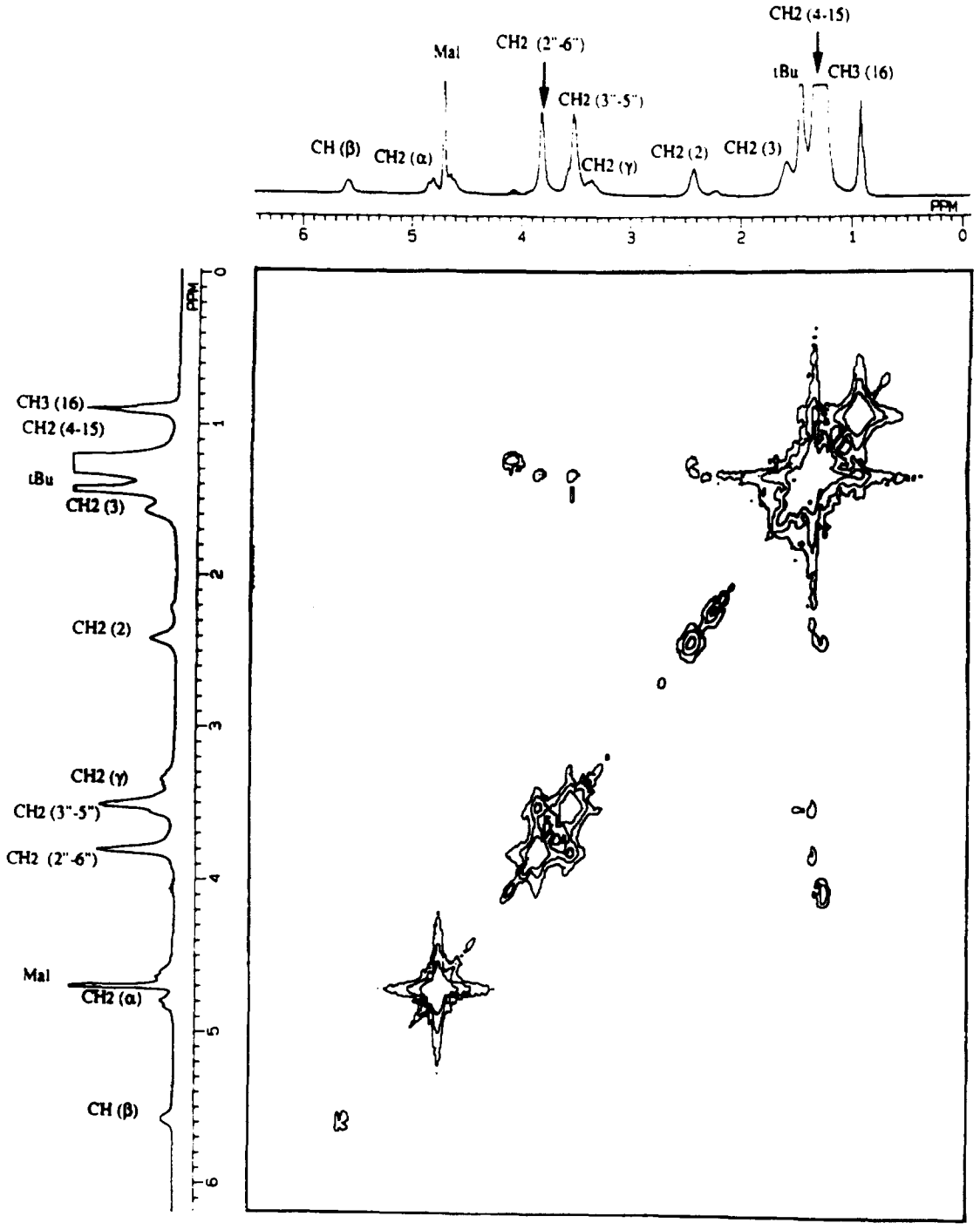

Fig. 4. NOESY (D_2O) spectrum of palmitoyl timolol malonate.

Table 1
Time in days for the prodrug concentration to decrease to half that of its original value (C)

	pH 3.5	pH 6.5	pH 7.5	pH 9.5
C^a = 0.3 mM/l μ = 0.1	9.6 ± 1.5	9.5 ± 3.7	11.8 ± 3.6	30.7 ± 3.1
C = 0.3 mM/l μ = 0.5	nd	>81	nd	nd
C = 7.9 mM/l μ = 0.1	nd	4.9 ± 0.4	nd	nd

nd, not determined.

matic hydrolysis was observed to be faster than pure chemical hydrolysis. After ca. 330 min, 50% of the prodrug was hydrolysed. This figure highlights the importance of esterases in the hydrolysis of palmitoyl timolol malonate. In a publication by Lee (1983), the author studied the localization of esterases (essentially acetylcholinesterases and butyrylcholinesterases) in different ocular segments and demonstrated that esterase activity was highest in the iris-ciliary body, followed by the cornea and the aqueous humor. Esterase activity in the cornea and aqueous humor was 50% and 2–5% with respect to that found in the iris-ciliary body. Therefore our study, performed with corneal homogenates, only represents one contribution of the total enzymatic hydrolysis that should occur in the entire ocular area, including the iris and ciliary processes.

The key role of the esterases in the hydrolysis of the prodrug must be taken into account together with previously published data (Pech et al., 1993). On the basis of a corneal permeation assay, using a Camber perfusion apparatus (Camber, 1985), no prodrug was found in the receiving solution thus indicating that the amount of timolol found in the receptor compartment originated from hydrolysis of palmitoyl timolol malonate. When compared with timolol maleate, only a relatively small amount of timolol permeated the corneal tissues after application of palmitoyl timolol malonate. But conversely, in the betamethasone-induced ocular hypertension model, the prodrug produces a significant decrease of intraocular pressure at prolonged time periods, as compared to the timolol maleate. These results could be explained by a prolonged pharmacological activity originating from a sustained release of the β-blocker, generated from corneal deposits of the prodrug. This assumption would support the absence in the receiving compartment (corneal permeation assay) and the prolonged pharmacological activity (in vivo assay). The presence of esterases in the corneal tissues is consistent with this assumption.

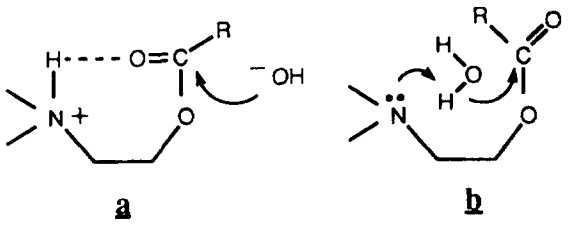

Fig. 5. Mechanisms of hydrolysis of palmitoyl timolol malonate.

4. Conclusions

After completing the synthesis of palmitoyl timolol malonate, we have shown its amphiphilic character through the use of nOe spectroscopy. The polarity of deuteriated water causes an extension of the oleofinic chain of the molecule. Hydrolysis studies have shown that the prodrug is very stable in aqueous solutions and that intramolecular aminolysis does not occur. The hydrolysis in corneal homogenates demonstrated the importance of the role of esterases in the regeneration of timolol from the prodrug and may explain our previously published in vivo results.

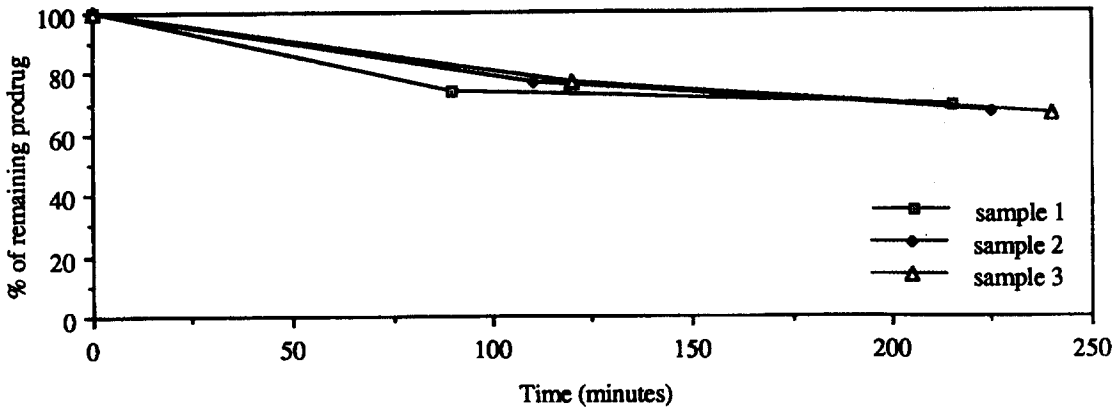

Fig. 6. Prodrug hydrolysis in corneal homogenates.

Acknowledgements

We thank Dr. Michael Lynch for his helpful suggestions.

References

Bangham, A.D., Standish, M.M. and Watkins, J.C., Diffusion of swollen lipids. *J. Mol. Biol.*, 13 (1965) 226–228.

Bundgaard, H., Buur, A., Chang, S.C. and Lee, V.H.L., Prodrugs of timolol for improved ocular delivery: synthesis, hydrolysis kinetics and lipophilicity of various timolol esters. *Int. J. Pharm.*, 33 (1986) 15–26.

Buur, A., Bundgaard, H. and Lee, V.H.L., Prodrugs of propranolol: hydrolysis and intramolecular aminolysis of various propranol esters and an oxazolidin — 2 — one derivative. *Int. J. Pharm.*, 42 (1988) 51–60.

Camber, O., An 'in vitro' model for determination of drug permeability through the cornea. *Acta Pharm. Suec.*, 22 (1985) 335–342.

Jordan, C.G.M., Quickley, J.M. and Timoney, R.F., Synthesis, hydrolysis kinetics and lipophilicity of O-acyl esters of oxprenolol. *Int. J. Pharm.*, 84 (1992) 175–189.

Lee, V.H.L., Esterase activities in adult rabbit eyes. *J. Pharm. Sci.*, 72(3) (1983) 239–244.

Maksay, G., Tegyey, A., Simon-Trompler, E. and Otvos L., Structural parameters in the microsomal hydrolysis of 3-acyloxy-1,4-benzodiazepines and the multiplicity of the esterases involved. *Eur. J. Drug. Metab. Pharmacokinet.*, 5 (1980) 193–200.

Nelson, W.L., Fraunfelder, F.T., Sills, J.M., Arrowsmith, J.B. and Kuritsky, J.N., Adverse respiratory and cardiovascular events attributed to timolol ophthalmic solution, 1978–1985. *Am. J. Ophthalmol.*, 102 (1986) 606–611.

Pech, B., Chetoni, P., Saettone, M.F., Duval, O. and Benoit, J.P., Preliminary evaluation of a series of amphiphilic timolol prodrugs: possible evidence for transscleral absorption. *J. Ocular Pharmacol.*, 9 (1993) no 2, 141–150.

Pech, B., Bouligand, Y., Proust, J.E. and Benoit, J.P., Supramolecular organization and thermal analysis of a prodrug of timolol: palmitoyl timolol malonate. *Proceed. 1st World Meeting APGI/APV, Budapest*, (1995) p. 129.

Tojo, K., Desai, D.S. and Chien, Y.W., Bioconversion of ocular prodrugs across rabbit cornea in vitro. *Proc. Int. Symp. Control. Rel. Bioact. Mater.*, 15 (1988) 133–134.