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Preparation and evaluation in vitro of solutions and o/w microemulsions containing levobunolol as ion-pair

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Summary

Aqueous and aqueous-PEG 200 solutions, and o/w microemulsions containing levobunolol (LB) coupled to octanoic acid (OA) as lipophilic ion-pair were submitted to a preliminary investigation in vitro, in view of possible ophthalmic applications. Evaluation of the following was carried out: *n*-octanol/water partition coefficient, permeation through an artificial lipophilic membrane and through hairless mouse skin, isotonicity and eye irritation in albino rabbits. Permeation studies in aqueous and in aqueous-PEG 200 solutions through the artificial membrane indicated a higher apparent lipophilicity of LB-OA with respect to the drug alone. The transport rate of LB-OA through hairless mouse skin was lower from a microemulsion containing 0.5% w/w drug than from a water-PEG 200 solution simulating the continuous phase, thus suggesting a possible reservoir effect of the dispersed phase of the microemulsion. The microemulsion, which was isotonic and non-irritating on rabbit eyes, appears as a potentially interesting ophthalmic vehicle for LB.

Introduction

Previous investigations were aimed at increasing the topical ocular bioavailability and at prolonging the release of timolol, a widely used β-blocking agent (Gallarate et al., 1988; Gasco et al., 1989). Analysis of the aqueous humour of rabbits receiving topical timolol coupled to octanoic acid as lipophilic ion-pair, both in solution and in o/w microemulsions, showed that the bioavailability of the drug from these systems was 3.5- and 4.2-times greater, respectively, than that resulting from administration of timolol alone (Gasco et al., 1989).

The aim of the present study was to extend the previous findings to levobunolol (LB), a potent, non-selective β-blocking agent used for topical treatment of increased intraocular pressure in patients with chronic open angle glaucoma or ocular hypertension. Even if the octanol/water partition coefficient of LB is higher than that of timolol (Schoenwald and Huang, 1983), formulation of the drug as an ion-pair might further increase its lipophilicity, and improve its ocular penetration. It was also desired to evaluate the

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possibility of achieving modulation of release by incorporating LB ion-pairs in an o/w microemulsion. Adverse reactions to topical LB (blepharitis, conjunctivitis, inflammation, itching and burning), have been observed in some cases (Gonzalez and Clissold, 1987). Administration of the drug in alternative vehicles, producing the same pharmacological effect with a lower dose, might possibly result in reduction of the side-effects.

Materials and Methods

Materials

Levobunolol (LB) hydrochloride was kindly supplied by Allergan S.p.A. (Pomezia, Rome); LB base was prepared from LB-HCl. The following materials were used as received: octanoic acid (OA), isopropyl myristate (IPM), hexanoic acid, 1-octanol and 1-dodecanol (Merck), ethyl oleate (Carlo Erba), capric-caprylic triglyceride (CCT, Myritol 318, Henkel KG & A), soya phosphatidylcholine 95% (SPC, Epikuron 200, Lucas Meyer), PEG 200 (Aldrich), sodium cholate (Sigma), RS 40 membrane and hydrophilic barrier foil (Sartorius). Monobutyrylglycerol (b.p. 139–140°C, 4 mmHg, $n^{20} = 1.4533$ (Schuette and Hale, 1930)) and sodium octyl phosphate (Brown et al., 1955) were prepared in our laboratory.

Apparatus

The following equipment was used: a Model 4050 spectrophotometer (LKB), capillary viscometer (Schott Geräte), HPLC apparatus consisting of a UV detector (LC 90), pump control unit (LC 250) and data station equipped with OMEGA 2 software (Perkin Elmer), and an optical microscope (Leitz Labovert).

Methods

HPLC determination of LB

The analysis of LB was carried out using a partially modified method described in the literature (Richman and Tang-Liu, 1990), employing the following. Column, Spherisorb C18 5 μm (15 cm · 4.5 μm); mobile phase, CH_3OH-0.027 M sodium heptanesulfonate + 0.027 M Na_2SO_4 in water (60:40), brought to pH 3.5 with H_2SO_4; flow rate, 1 ml/min; UV detection, 257 nm; retention time, 3.3 min.

Levobunolol-octanoic acid (LB-OA) ion-pairs in aqueous solutions

Partition studies: Partitioning of LB in the presence of increasing concentrations of OA was evaluated using pH 7.4 and 6.5 phosphate buffers saturated with *n*-octanol, and *n*-octanol saturated with buffer. The apparent partition coefficients were determined by shaking at 25°C, until a distribution equilibrium was reached, equal volumes of *n*-octanol and buffer solutions containing LB alone (0.5×10^{-4} M) or LB in the presence of increasing concentrations of OA (0.5×10^{-4}–1.0×10^{-3} M).

Permeation through artificial membranes: The apparatus used for permeation studies has been described in a previous paper (Gallarate et al., 1988). Briefly, the membrane consisted of a hydrophilic foil (soaked in water until swollen) and an RS-40 membrane filter impregnated with dodecanol, pressed together to form a hydrophilic/lipophilic double barrier. The donor and receiving compartments of the permeation cell contained equal volumes of solution; sink conditions were secured by the presence of HCl (0.1 N) in the receiving compartment. All experiments were carried out at 25 ± 0.1°C.

The permeation studies were performed at pH 7.4 and 6.5, using solutions containing LB alone or LB plus OA at different molar ratios (1:1–1:20). The concentration of LB was always 3.0×10^{-4} M. To avoid a possible influence of anions on the formation of ion-pairs, the pH of the diffusing solution was adjusted by adding hydrochloric acid or sodium hydroxide. At appropriate intervals an aliquot of the receiving solution was withdrawn for UV analysis (257 nm, $\log \epsilon = 3.97$). The permeability constant, K_d, was calculated from the equation reported previously (Gallarate et al., 1988).

LB-OA ion-pairs in o/w microemulsions

Preparation: OA was a component of the oily phase of all microemulsions. Microemulsions 1–4 were prepared as follows: lecithin was suspended

in water; the second surfactant (sodium octyl phosphate or sodium cholate) was then added, achieving partial solubilisation. A coarse emulsion was then obtained by addition of oil. A cosurfactant (hexanoic acid or monobutyrylglycerol) was then added until a transparent system was obtained. Microemulsion 5 was prepared by suspending lecithin in a water-PEG (3.18 : 1 w/w) solution, then adding oil to form a coarse emulsion: clarity was obtained by adding the required amount of sodium cholate. LB was then added to each microemulsion to a final concentration of 0.5% w/w.

Composition: The following formulations, whose pH and tonicity were in a suitable range, were selected after a series of preliminary trials:

(1) SPC, 5.7%; sodium octyl phosphate, 3.9%; H_2O, 70.8%; hexanoic acid-sodium hexanoate, 6.6%; IPM + OA (10% w/w), 9.3%; CCT, 3.2%; LB, 0.5% (pH 6.5).

(2) SPC, 9.2%; sodium octyl phosphate, 3.6%; H_2O, 65.7%; monobutyrylglycerol, 9.3%; ethyl oleate + OA (10% w/w), 8.7%; CCT, 3.0%; LB, 0.5% (pH 6.5).

(3) SPC, 8.4%; sodium cholate; 3.5%; H_2O, 69.2%; hexanoic acid-sodium hexanoate, 6.3%; ethyl oleate + OA (10% w/w), 9.1%; CCT, 3.0%; LB, 0.5% (pH 6.3).

(4) SPC, 8.7%; sodium cholate, 3.6%; H_2O, 71.8%; hexanoic acid- sodium hexanoate, 2.8%; ethyl oleate + OA (10% w/w), 9.4%; CCT, 3.2%; LB, 0.5% (pH 6.4).

(5) SPC, 6.2%; sodium cholate, 3.7%; H_2O, 62.0%; PEG 200, 19.5%; ethyl oleate + OA (10% w/w), 8.1%; LB, 0.5% (pH 6.75).

Isotonicity studies: The microemulsions were tested for isotonicity by observing the behaviour of human erythrocytes suspended in them. A droplet of human blood was added to a drop of microemulsion, and the occurrence of hemolysis or marked changes in the appearance of erythrocytes was evaluated by optical microscopy (magnification = 12.5 × 63).

Determination of the apparent partition coefficient of LB (P_{cos}) among all microemulsion components except lecithin: The method used to determine the apparent partition coefficient of LB (P_{cos}) has been described previously (Trotta et al., 1989). Aqueous or water-PEG 200 (microemulsion 5) solutions of LB, buffered at the appropriate pH for each microemulsion were used; the amount of levobunolol corresponded to the concentration in the microemulsion (0.5% w/w). Ethyl oleate (or IPM), the cosurfactant (hexanoic acid or monobutyrylglycerol) and the surfactant (octyl phosphate or sodium cholate) were then added to the aqueous solution. The same ratios (w/w) of the components as present in the microemulsions were maintained. The mixtures were shaken at 25°C until equilibrium was reached; after centrifugation the concentration of LB in the aqueous phase was determined by HPLC.

Eye irritation studies: The microemulsions under study were tested for ocular tolerability and irritation on male albino New Zealand rabbits (2.5–3.0 kg); each preparation was tested at least on three animals. All eyes were examined prior to testing to exclude any existing ocular irritation. At $t = 0$, 50 μl of the vehicle were applied in the lower conjunctival sac: the eyes were examined 5, 30, 60 and 120 min after application. Observation of injuries was made on the bulbar and palpebral conjunctivae, and the irritation was evaluated according to a scoring scale reported in a previous paper (Bottari et al., 1978).

Permeation through artifical membranes: The permeation of LB from microemulsion no. 5 (molar ratio OA/LB = 3.45 : 1; molar ratio water:PEG = 3.18 : 1) was studied using the method described for aqueous solutions.

Permeation through hairless mouse skin: The permeation of LB from microemulsion no. 5 through full thickness abdominal skin of male hairless mice aged 4–6 weeks was investigated at 25°C using the apparatus described before. The receptor cell was filled with 0.1 N pH 7.4 phosphate buffer; samples of this solution were withdrawn for HPLC analysis at appropriate intervals, and replaced with fresh buffer.

LB ion-pairs in water-PEG solutions

Preparation: Ion-pairs of LB and OA were prepared in water-PEG 200 solutions. The following systems were examined: LB 0.1% + OA in water-PEG solution (3.18 : 1 w/w), pH 6.75. Molar ratio, OA/LB 3.45 : 1 (the same as in microemulsion no. 5); LB 0.4% + OA in water-PEG

solution (1 : 1 w/w), pH 7.4. Molar ratio, OA/LB 3.45 : 1.

Permeation through artificial membranes: The permeation of LB ion-pairs in water-PEG 200 solutions was studied as described for aqueous solutions and for microemulsions. The following reference solutions were used: LB 0.5% w/w in water-PEG (3.18 : 1) solution, pH 6.75; LB 0.4% w/w in water-PEG (1 : 1) solution, pH 7.4.

Permeation through hairless mouse skin: These experiments were performed as described previously.

The examined systems were: LB 0.1% + OA in water-PEG 200 (3.18 : 1 w/w) solution, pH 6.75. Molar ratio OA/LB, 3.45 : 1; LB 0.4% + OA in water-PEG 200 (1 : 1 w/w) solution, pH 7.4. Molar ratio, OA/LB 3.45 : 1.

The following reference solutions were used: LB 0.5% w/w in water-PEG 200 (3.18 : 1) solution, pH 6.75; LB 0.4% w/w in water-PEG 200 (1 : 1) solution, pH 7.4.

Results and Discussion

One of the first aims of the investigation was to verify if formation of ion-pairs with octanoic acid (OA) would increase the lipophilicity of levobunolol (LB). The apparent partition coefficients (P_{app}) of LB between octanol and buffer, determined at two different pH values (6.5 and 7.4) in the presence of increasing amounts of OA are reported in Table 1. An increase in P_{app} with increasing molar ratio OA/LB, greater at pH 6.5 than at pH 7.4, is apparent from the data.

The increase in the apparent lipophilicity of LB resulting from the formation of ion-pairs was also evaluated in experiments in which the drug was allowed to permeate through an artificial, hydrophilic-lipophilic membrane. The permeability coefficients (K_d) of LB from solutions, determined at pH 7.4 and 6.5, are reported in Table 2. The K_d values increased with increasing OA concentration, at both pH values. Permeability coefficients lower than expected were found at pH 7.4, at LB/OA molar ratios 1 : 20 and 1 : 30. This was proven to be a consequence of uptake of the ion-pair by the lipophilic membrane. In Fig. 1, the values of log K_d at pH 6.5 are reported vs log P_{app} at the same pH. The linear relationship existing between these parameters confirms that an increased amount of the lipophilic ion-pair produced an enhancement of the rate of diffusion through the membrane.

TABLE 1

Apparent partition coefficients octanol / phosphate buffer (P_{app}) of levobunolol in the presence of increasing concentrations of octanoic acid

Molar ratio LB:OA	P_{app}	
	pH 7.4	pH 6.5
LB alone	2.52	0.30
1:1	2.80	0.50
1:5	4.18	0.81
1:10	5.77	1.22
1:20	6.53	1.56
1:30	8.16	–
1:50	9.43	–

As a consequence of the lipophilicity increase, the amount of LB dissolved in water in the presence of 10-times its amount of OA was 0.015% w/w at pH 6.5. Since this concentration is too low for ocular administration, the possibility of developing suitable o/w microemulsions, containing an adequate drug load, was considered. Previous studies have shown that such microemulsions, whose dispersed phase acts as a drug reservoir for lipophilic ion-pairs, can constitute potentially useful ocular delivery systems (Gasco et al., 1988; Trotta et al., 1989).

TABLE 2

Permeability coefficients through an artificial membrane (K_d) of levobunolol in aqueous solution in the presence of octanoic acid at increasing molar ratios

Molar ratio LB:OA	K_d (cm s^{-1})	
	pH 7.4	pH 6.5
1:0	2.0×10^{-5}	1.1×10^{-5}
1:1	2.3×10^{-5}	1.8×10^{-5}
1:5	3.8×10^{-5}	3.0×10^{-5}
1:10	4.9×10^{-5}	4.1×10^{-5}
1:20	4.6×10^{-5} [a]	4.9×10^{-5}
1:50	3.5×10^{-5} [a]	–

[a] Uptake by the membrane.

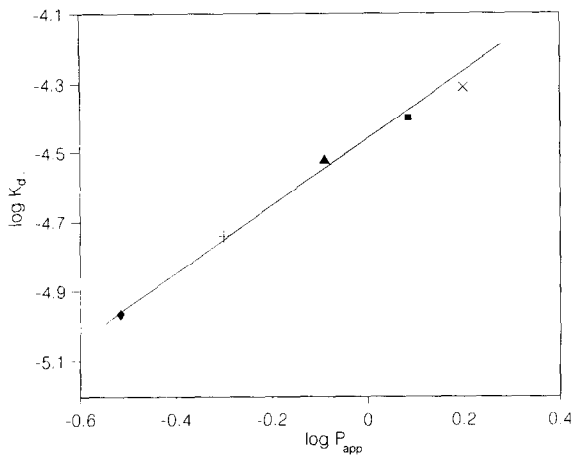

Fig. 1. Log permeability coefficients vs log apparent octanol/water partition coefficients of levobunolol (LB) at pH 6.5, in the absence and presence of octanoic acid (OA) at increasing molar ratios. (♦) LB alone; (+) LB/OA 1:1; (▲) LB/OA 1:5; (■) LB/OA 1:10; (×) LB/OA 1:20.

Several biocompatible, non-toxic substances were tested as surfactants and cosurfactants for the microemulsions, in an attempt to develop an optimal system for ocular delivery. It was desired not only to obtain an adequate drug reservoir in the dispersed phase, but also to ensure a sufficiently fast release of the drug, to counteract the rapid elimination of the medication from the eye surface.

The vegetable oils usually employed in ocular preparations are esters (Dolder, 1990), and the partitioning between esters and water is usually rather low for β-blockers and other therapeutic agents. In the present microemulsions, OA was used as a counterion in order to obtain better partitioning of LB into the dispersed phase. A final series of microemulsions (1–5) was prepared, and the apparent partition coefficient (P_{cos}) of LB between water and all components of the microemulsions except lecithin, roughly indicative of the partitioning of the drug among the microemulsion phases, was determined (Trotta et al., 1989). The P_{cos} values of microemulsions 1–5 were 2.5, 2.2, 2.0, 2.2 and 1.1, respectively. The P_{cos} of LB between ethyl oleate and water without OA at pH 6.75 was always 0.6. It can be noted that the value of P_{cos} increased from 0.6 in the absence of OA up to a maximum of 2.5. The total amount of dissolved levobunolol was in all cases 0.5% w/w.

Isotonicity and tolerability tests carried out on the microemulsions, the results of which are summarized in Table 3, showed that microemulsions 1 and 3 were hypertonic, and microemulsions 2 and 4 were irritating. Consequently, they were abandoned. Microemulsion 5, containing lecithin as surfactant, ethyl oleate as oil, and a water-PEG 200 mixture as hydrophilic phase (pH 6.75), in contrast, was isotonic and non-irritating. This was evidently due to the presence of sodium cholate as cosurfactant: a 5% aqueous solution of this compound was shown in separate tests to be well tolerated by rabbit eyes.

The value of P_{cos} for microemulsion 5 was lower (1.1) than those of microemulsions 1–4: this effect can be ascribed to the presence of a water-PEG 200 (3.18:1) mixture, in which LB and its ion-pair partitioned differently from water alone.

At pH 6.75, the permeability coefficient (K_d) of LB from microemulsion 5 through the double membrane (2.0×10^{-6} cm s^{-1}) was lower than that observed with an aqueous solution (1.1×10^{-5} cm s^{-1}), and in the same range as that observed with a water-PEG 200 solution (2.2×10^{-6} cm s^{-1}) at the same pH (cf. Table 4). The water-PEG 200 ratio in the latter solution was the same as in the external phase of the microemulsion, even if, in the microemulsion, part of PEG 200 might be at the interphase and

TABLE 3
Results of isotonicity tests and of tolerance studies in albino rabbit eyes

Microemulsion no.	Tonicity	Tolerance
1	hypertonic	n.t.
2	weakly hypotonic	severely irritating
3	hypertonic	n.t.
4	isotonic	mildly irritating
5	isotonic	nonirritating

n.t., not tested.

TABLE 4

Permeation data of levobunolol, alone and as ion-pair, from different media through an artificial membrane and through hairless mouse skin

Permeant	Diffusion medium (H_2O or H_2O:PEG 200)	pH	[Levobunolol] (% w/w)	K_d (cm s^{-1}) (double membrane)	% permeated after 5 h (hairless mouse skin)
LB alone	H_2O	6.75	0.008%	1.1×10^{-5}	–
LB alone	H_2O	7.40	0.008%	2.0×10^{-5}	–
Microemulsion 5	3.18:1 [a]	6.75	0.5%	2.0×10^{-6}	2.6
LB alone	3.18:1 [a]	6.75	0.5%	2.2×10^{-6}	1.1
LB alone	1:1 [b]	7.40	0.4%	6.2×10^{-7}	2.1
LB + OA	3.18:1 [a]	6.75	0.1%	1.5×10^{-5}	3.9
LB + OA	1:1 [b]	7.40	0.4%	3.7×10^{-6}	4.5

[a] Viscosity of medium at 25°C = 1.2 cP; apparent partition coefficient of ILB between dodecanol and water-PEG 200 (3.18:1) at 35°C (pH 7.4) = 0.46.

[b] Viscosity of medium at 25°C = 6.4 cP; apparent partiton coefficient of LB between dodecanol and water-PEG 200 (1:1) at 35°C (pH 7.4) = 0.23; between dodecanol and water = 1.7.

possibly in the dispersed phase. Therefore, the permeability coefficient of levobunolol from water-PEG 200 media (solution or microemulsion) was about 5-times lower than that from an aqueous solution. These results emphasize the fact that the medium can greatly affect the permeability coefficient of LB. The effect can be ascribed to: (a) a different diffusion rate of LB in dissimilar media; (b) different partitioning of the drug between dodecanol (present in the lipophilic membrane) and the two media (cf. footnote in Table 4); and (c) the different viscosity of the two media.

Permeation experiments were then performed at two different pH values, using water-PEG solutions of LB (with two different water/PEG 200 ratios) in the presence of OA. As shown in Table 4, the diffusion rate of the ion-pair through the double membrane was about 6-times greater than that of LB alone, and increased further with increasing amount of PEG 200 present in the solution, and the pH. The latter conditions, in contrast, decreased the K_d of levobunolol alone. The maximum amount of LB dissolved as ion-pair was 0.1% at pH 6.75 and 0.4% at pH 7.4.

In view of the problems posed by the double synthetic membrane, further permeation experiments were carried out using hairless mouse skin, and different permeant-counterion-permeation medium combinations: the results are reported in Table 4. In water solution and in the absence of counterion, the concentration of LB in the donor solution was very low (0.008%), and no permeation was observed at both pH 6.75 and 7.40. Higher LB concentrations could be obtained in microemulsion 5, and in water-PEG solutions, containing the drug alone or as ion-pair. The

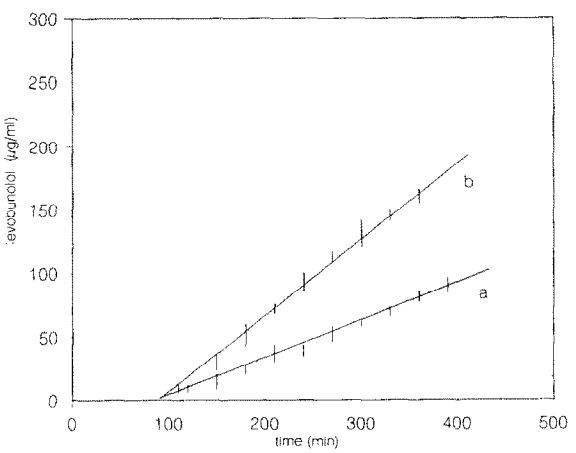

Fig. 2. Levobunolol (LB) permeated vs time through hairless mouse skin from water-PEG (1:1) solution at pH 7.4 in the absence (curve a), and presence (curve b) of octanoic acid (OA). Molar ratio, LB/OA = 1:3.45. The LB concentration in both cases was 0.4% w/w.

amount of LB diffused vs time from these systems was higher when the permeant was present as an ion-pair than when it was alone. The percentages of LB permeated through the skin after 5 h at pH 6.75 from microemulsion 5 (drug concentration, 0.5%), and from a 3.18:1 water-PEG solution containing the ion-pair (drug concentration, 0.1%) at the same pH were 2.6 and 4.1, respectively. The smaller amount of drug permeated in time from the microemulsion with respect to the solution might indicate the presence of a drug reservoir in the dispersed phase. In the absence of counterion, the percent LB permeated after 5 h from the same water-PEG solution was 1.1, even when the drug concentration was higher (0.5%). Thus, the presence of the counterion increased the skin permeation of LB about 4-times in the case of the solution, and over 2-times in the case of the microemulsion.

The results of skin permeation experiments carried out at pH 7.4 on water-PEG 1:1 solutions containing 0.4% LB, alone and in the presence of OA (molar ratio 3.45:1) are also reported in Table 4, and, in graphical form, in Fig. 2. As indicated in Table 4, the percent LB diffused after 5 h showed a 2-fold increase (from 2.1 to 4.5) in the presence of the counterion.

In conclusion, although the present results need further validation with transcorneal permeation experiments in vitro and with studies in vivo, they appear to indicate that the permeation of levobunolol through a biological membrane can be greatly enhanced by formulating the drug as an ion-pair with octanoic acid. Incorporation of the ion-pair into a properly formulated microemulsion can provide a reservoir effect, potentially useful in ocular delivery. It is hoped that studies on experimental animals, now under way, will confirm the present preliminary experimental data.

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