Comparison of Azone and Hexamethylene Lauramide in Toxicologic Effects and Penetration Enhancement of Cimetidine in Rabbit Eyes

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INTRODUCTION

Two penetration enhancers, Azone and hexamethylene lauramide, were evaluated for their potential applications in improving ocular therapy. Evidence from *in vitro* studies indicates that Azone produces dramatic to nil penetration enhancement effects on the corneal penetration of drugs varying in lipophilicity (1). The corneal drug absorption of poor penetrators such as cimetidine and acetazolamide benefit most from the coadministration of the enhancers *in vitro*. In the corneal chamber studies, drugs and the enhancer were coincubated with the corneal epithelial surface and the appearance of drug in the receptor chamber over time was monitored. Four enhancers [Azone, hexamethylene lauramide (HML), octamethylene lauramide, and decylmethyl sulfoxide) were investigated (1). Increased corneal hydration was invariably noted when the enhancer was present in the corneal chamber studies. Therefore, the pronounced penetration enhancement observed previously could be due to the compromised epithelial structure as part of the adverse effects of the enhancer on the cornea.

Because the chamber studies *in vitro* involved prolonged exposure of the enhancer to the cornea, the penetration enhancement observed *in vitro* might not be predictive of its potential use in topical instillations to eyes. Data generated in our laboratory, *in vivo* and *in vitro*, on the effect of Azone on levobunolol corneal absorption lacked an apparent correlation. Statistically significantly enhancement in ocular absorption of levobunolol could not be demonstrated *in vivo* using a low concentration (0.025%) (2) and higher concentrations (0.05 and 0.1%, unpublished results) of Azone. However, a 120% increase in the corneal permeability coefficient of bunolol was noted (1). As a result, cimetidine, with a log P value of 0.4, was chosen in this study to investigate the relationship between *in vitro* and *in vivo* enhancement effects for Azone and HML. In this report, the ocular adverse effects are summarized for Azone and its structural analogue, hexamethylene lauramide. The penetration enhancement effects of Azone and HML on the ocular absorption of cimetidine *in vivo* were also presented.

MATERIALS AND METHODS

^{14}C-Cimetidine (labeled at the *cyano* position) with a specific activity of 20 μCi/mg was obtained from SK&F Labs, Welwyn Gardens, England. Azone (1-dodecylhexahydro2H-azepin-2-one) was obtained from Nelson Research, Irvine, CA, and hexamethylenelauramide (hexahydro-1-lauroyl-1H-azepine; HML) was synthesized in-house. The chemical purity of Azone and HML was greater than 99%. All other chemicals used in this study were reagent grade or highest grade possible.

This study employed adult female New Zealand albino rabbits, weighing approximately 2 to 4 kg. All animals were selected from our colony in good health with clinically normal eyes. The ocular status in each animal was established prior to the study. All instillations in the toxicologic studies were made in the left eye with the right eye remaining untreated as a control. All rabbit eyes, treated and untreated, were grossly examined for obvious ocular changes at the time of each eyedrop (~50 μl) instillation (3).

The objective of the safety evaluation studies was to establish a range of safe doses for clinical testing and hence the enhancer concentrations used in the toxicological studies were in some cases much higher than those used in the ocular absorption studies. Discomfort was graded on a basis of severity and duration. The severity could be slight, characterized by intermittent blinking and/or squinting, to severe, characterized by firm closure of the eye for prolonged periods of time with repeated pawing. The duration of discomfort was also recorded and could vary from seconds to minutes. When more than one type of discomfort was observed, the scores recorded were the greatest severity of discomfort observed and the duration of that type of discomfort. Irritation including hyperemia, chemosis, and mucoidal discharge was graded on severity alone. Cytotoxicity was graded according to severity and area of involvement and was compared to the initial observations made prior to the study initiation. The rabbit eyes were also examined with the aid of a slit-lamp microscope with fluorescein and rose bengal stainings before, during, and after the study. Grading of the microscopic findings was based upon the method of McDonald and Shadduck (4). Direct ophthalmoscopy was conducted prior to the start and at the end of the study. All toxicologic studies were performed as open-label studies to ensure that the test formulations were correctly instilled.

Outlines of the toxicologic studies are as follows.

(1) Three rabbits received one drop of 0.1% Azone ophthalmic emulsion (containing ethoxyol 24, EDTA disodium, and BAK) 16 times a day for 2 days at half-hour intervals.

(2) Six rabbits were divided into two treatment groups (three per group) and received one drop of 0.1%

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Azone or the preserved saline vehicle (containing ethoxyol 24, EDTA disodium, and BAK) 16 times in 1 day.

(3) Twenty-one rabbits were divided into seven treatment groups and received one drop of the test formulations 16 times in one day. The test formulations were 0.7, 0.4, and 0.1 Azone or HML and the placebo vehicle. Those rabbits exhibiting positive ocular findings at the last dose were examined again approximately 17 hr following the last dose and on a daily basis thereafter until no reactions were observed.

(4) Eighteen rabbits, three in each treatment group, were administered one drop of the test formulation (0.7, 0.4, and 0.1% of Azone or HML) eight times in 1 day at hourly intervals. The rabbits which exhibited corneal staining at the last examination were examined again approximately 17 hr following the last dose.

(5) Twenty-four rabbits (six in each group) underwent one of the following treatments: 0.1% Azone eight times daily at hourly intervals, 0.1% Azone four times daily at 2-hr intervals, 0.1% Azone two times daily at 4-hr intervals, and the placebo vehicle eight times daily at hourly intervals. The study duration was 28 days for all animals. At the end of the study, rabbits were euthanized. The ocular tissues were excised and preserved in 10% buffered formaldehyde. Before histopathologic evaluation, the tissues were trimmed, sectioned at 6 μm, and stained with hematoxylin and eosin. The globe was sectioned in a midplane from the central cornea through the optic nerve. The lens was sectioned *in situ*.

(6) Using the same study design as described under study 5, the effects of 0.1% HML were evaluated.

In the ocular absorption study, each rabbit received one eyedrop of 1% ^{14}C-cimetidine with 0, 0.025, 0.1, 0.25, 0.5, or 1% of the enhancer, Azone or HML, formulated in phosphate-buffered saline. At various times (i.e., before dosing and 0.5, 1, 2, 3, and 4 hr postdosing), the animals were euthanized and the aqueous humor was collected for drug analysis. The radioactivity content was assayed directly by liquid scintillation counting and was calculated as micrograms of cimetidine per milliliter of aqueous humor. Four eyes were used at each sampling time per treatment group. The area under the aqueous humor cimetidine concentration–time curve for each treatment was calculated using the trapezoidal rule (2) and the Satterthwaite approximation from time 0 to 4 hrs postdosing.

Corneal hydration was measured as an indication of the effect of Azone treatment on corneal swelling. Corneas, with or without a 10-min topical application of Azone (0.01–0.25%), were freshly excised. The corneal hydration was also determined after a 4-hr incubation period in the corneal chamber device (5). The state of corneal hydration was measured by a thermogravimetric analyzer (between 50 and 180°C) and also by heating in an oven at 105°C to a constant weight. The corneal hydration was calculated as one minus the ratio of the dried and wet weights of cornea. The effect of HML on corneal hydration during the incubation period was reported elsewhere (1).

RESULTS

Findings of the toxicologic studies are summarized in Table I. In all animals treated with the placebo vehicle formulation, there was no ocular discomfort, no irritation to the conjunctiva, and no cytotoxicity to the cornea. HML at 0.7, 0.4, and 0.1% was discomforting to rabbit eyes. At the two higher strengths, HML was slightly irritating to the conjunctiva as evidenced by the observation of slight conjunctival hyperemia and some ocular discharge. HML was not cytotoxic to the cornea in all studies.

Azone at all three doses (0.7, 0.4, and 0.1%) in the 1-day acute test was slightly discomforting to eyes and irritating to conjunctiva. Corneal toxicity was observed for Azone after 0.7 and 0.4% doses 16 times in 1 day. After the last dose of 0.7% Azone, all eyes exhibited moderate congestion, slight discharge, slight flare, slight iritis, and slight to mild corneal opacity involving up to 75% of the cornea with severe fluorescein staining involving up to 50% of the cornea. The ocular toxicity was reversible by cessation of treatment with Azone and the corneal lesions disappeared after 6 days of observation.

In the 28-day subacute study with 0.1% Azone, slight ocular discomfort lasting 0.5 to 1 min postdosing was observed. Microscopic examination of eyes treated eight times daily revealed corneal epithelial thinning in the treated eye in two of six animals and the presence of apparent intranuclear inclusions within some corneal epithelial cells in one of six animals. Mild corneal epithelial thinning was observed in the left eye in one of six animals treated with 0.1% Azone four times daily and in the left eyes in two of six animals treated with 0.1% Azone twice daily. This change was not observed in the right eyes or in eyes treated with the placebo vehicle. The epithelial thinning in the eye treated four times daily appeared to be the result of both erosion, loss of superficial layers, and atrophy, thinning of existing layers (Fig. 1). It was limited to the central portion of the cornea. In eyes treated two times daily, the corneal thinning appeared to be the result of erosion. The underlying corneal stroma was histologically normal in all cases.

In the 28-day subacute study, 0.1% HML was found to be mildly discomforting to the eye but not irritating to the conjuctiva or toxic to the cornea. In the same study, the placebo vehicle was also found to be slightly discomforting to the eye but not irritating to the conjunctiva or toxic to the cornea of rabbit eyes. This placebo vehicle was the same one used in the 28-day subacute study with 0.1% Azone. Histopathologically, conjunctival squamous metaplasia was noted in the lower eyelids of the treated eyes in all of the 0.1% HML dose group and in one-third of the placebo group. This subtle change was recognized as the apparent reduction or loss of conjunctival mucous cells with the resultant conversion to nonkeratinizing stratified squamous epithelium of a thickness approximately equal to that of the normal epithelium. This subclinical change was most likely due to very subtle irritation not observable by gross or slit-lamp examination. There was no apparent associated inflammation. These subclinical changes were potentially reversible. All of the other changes observed histopathologically were not significant because they were observed in both treated and untreated eyes.

Table I. Summary of Ocular Toxicological Studies

Study No.	Treatment	Dosing regimen	Duration (days)	Ocular discomfort	Conjunctival irritation	Cytotoxicity
1	0.1% Azone	1gtt OS 16×/day[a]	2	No	No	Possible
2	0.1% Azone	1gtt OS 16×/day	1	No	No	No
	Placebo			No	No	No
3	0.7% Azone	1gtt OS 16×/day	1	Slight	Slight	Toxic
	0.4% Azone			Slight	Slight	Toxic
	0.1% Azone			Slight	Slight	No
	0.7% HML[b]			Moderate	Slight	No
	0.4% HML			Mild	Slight	No
	0.1% HML			Slight	No	No
	Placebo			No	No	No
4	0.7% Azone	1gtt OS 8×/day	1	No	No	Possible
	0.4% Azone			No	No	No
	0.1% Azone			No	No	No
	0.7% HML			Slight	No	No
	0.4% HML			Slight	No	No
	0.1% HML			No	No	No
5	0.1% Azone	1gtt OS 8×/day	28	Slight	No	Toxic
	0.1% Azone	1gtt OS 4×/day	28	Slight	No	Toxic
	0.1% Azone	1gtt OS 2×/day	28	Slight	No	Toxic
	Placebo	1gtt OS 8×/day	28	No	No	No
6	0.1% HML	1gtt OS 8×/day	28	Mild	No	No
	0.1% HML	1gtt OS 4×/day	28	Mild	No	No
	0.1% HML	1gtt OS 2×/day	28	Mild	No	No
	Placebo	1gtt OS 8×/day	28	Slight	No	No

[a] One drop in left eye 16 times a day.
[b] Hexamethylene lauramide.

The penetration enhancer concentration used in the *in vivo* absorption study was kept less than 0.1% to obviate any untoward effects of the enhancer on the eye. Both Azone and HML improved the ocular absorption of cimetidine, a poor penetrator. The temporal profiles of cimetidine concentration in aqueous humor in the absence and in the presence of the enhancer are shown in Fig. 2. The AUC values in the aqueous humor when cimetidine was instilled in the absence or in the presence of a penetration enhancer are listed in Table II. At 0.025% Azone or HML, the ocular bioavailabilities of cimetidine were increased 3.9- and 4.4-fold, respectively. At 0.1% concentration, both Azone and HML improved the ocular absorption of cimetidine, 22- and 10-fold, respectively. The penetration enhancer at a concentration greater than 0.1% did not appear to produce significantly higher ocular cimetidine absorption. The relationship between the enhancers' effects on the apparent corneal permeability coefficient (measured *in vitro*) and the aqueous humor area under the curve of cimetidine is depicted in Fig. 3 for both Azone and HML. Overall, a positive correlation between *in vitro* and *in vivo* measurements of cimetidine absorption across the cornea was demonstrated.

Normally in corneal penetration experiments *in vitro*, perfusing the cornea with dilute drug solutions in glutathione-buffered Ringer's (GBR) solution would not alter the normal hydration state of the cornea (i.e., 75–80% hydration). In this study, when the cornea was exposed to 0.25% Azone for 10 min, there was no immediate swelling. However, when the treated cornea underwent a 4-hr perfusion in GBR, there was a significant degree of corneal swelling and the cornea appeared slightly cloudy in the end. A 10-min exposure of the cornea to 0.01 or 0.05% Azone followed by GBR perfusion did not appear to cause similar swelling. The observed weight loss in the cornea upon heating was due primarily to the loss of water.

DISCUSSION

Both penetration enhancers examined in this study caused ocular discomfort and conjunctival hyperemia at higher dosing strengths (i.e., 0.4 and 0.7%). Their ocular adverse effects were dependent on the concentration of the enhancer, the dosing frequency, and the length of the exposure to the enhancer. After a low dose, less frequent dosing, and a shorter treatment period (e.g., 0.1% eight times in 1 day), there was no obvious untoward effects in eyes with either compound. Increasing the dosing frequency to 16 times in 1 day increased the incidence of ocular discomfort with both compounds and that of irritation to conjunctiva with Azone. As the dosing strength of HML increased, ocular discomfort also increased in incidence, severity, and duration in treated eyes. However, no cytotoxic effect in the cornea was observed in any eyes treated with HML. The study results indicate that corneal cytotoxic effects are produced by Azone at high doses (e.g., 0.4 and 0.7%) after 16 instillations in 1 day and at a low dose, 0.1%, following topical instillation 8 times daily for 28 days. Histopathological observation demonstrates corneal toxicity, as demonstrated by epithelial thinning, as a result of erosion and/or atrophy. The exact mechanism for the corneal cytotoxicity by Azone is unknown thus far.

Because large numbers of rabbits and test formulations

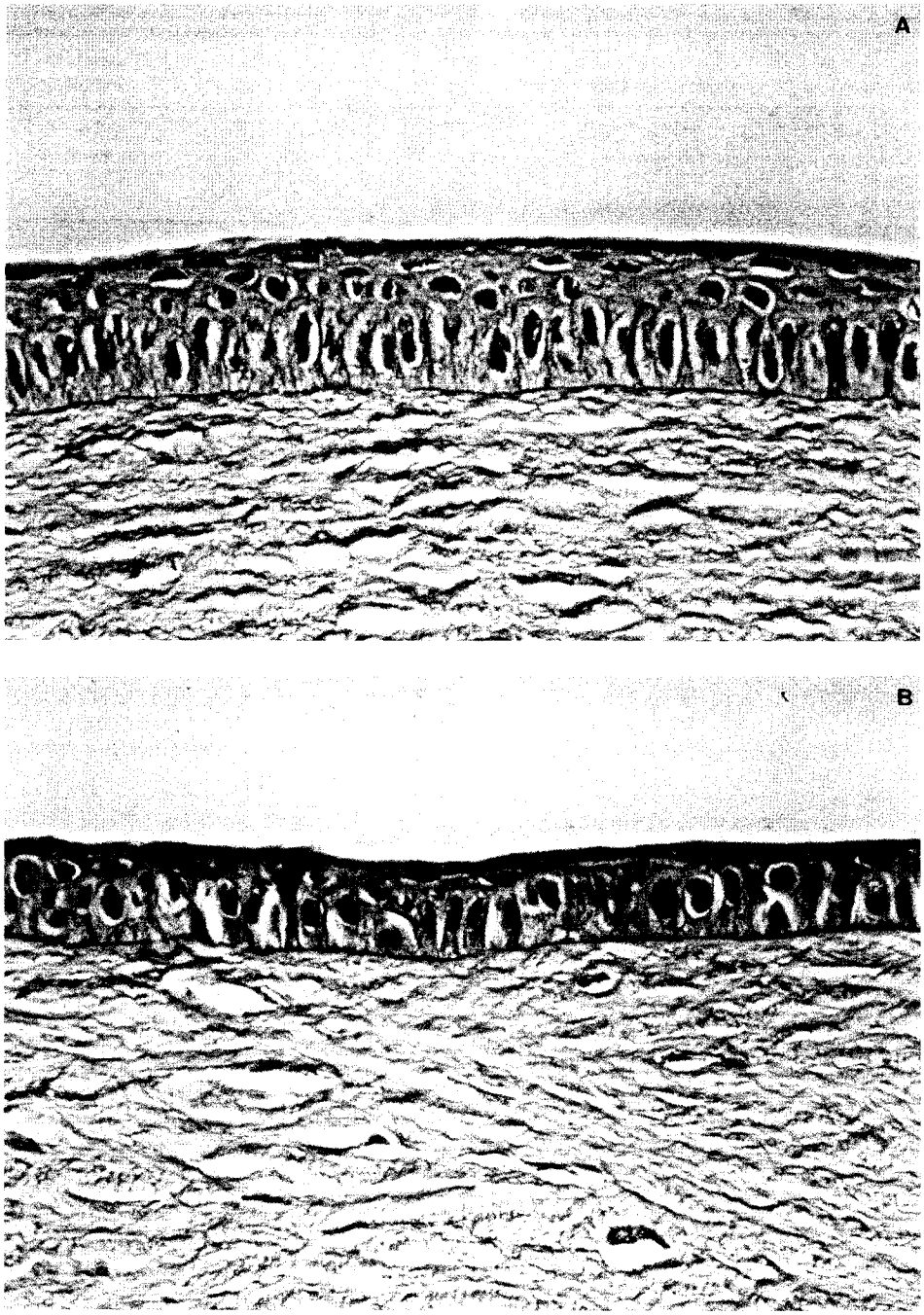

Fig. 1. Histopathological examination of the cross-section of cornea. (A) Cornea of the left eye of a rabbit treated with 0.1% Azone four times daily for 28 days. The epithelium appeared normal. (B) Cornea of the left eye of a rabbit treated with 0.1% Azone eight times daily for 28 days. There was epithelial thinning and erosion. 400×.

were tested concomitantly in the toxicological study protocols 5 and 6, we chose to use the open-label study design in order to ensure correct study execution. To keep the study design simple, there was no attempt to keep the treated eyes randomized between right and left eyes. This was also because normally no difference in study results would occur between eyes. The same person was responsible for both treatment and observation phases. The potential for observer bias was not judged to have a significant effect on the outcome of these studies, and additionally, all of the results were compared within each study. Slight study-to-study variations in scoring would be expected. This was demonstrated by the different scoring results in ocular discomfort for the placebo treatment groups. Nevertheless, HML did not produce cytotoxicity results in any of the studies.

The magnitude change in the enhancement of ocular cimetidine absorption was similar between *in vitro* and *in vivo* experiments (Fig. 3). That is, more than one order of

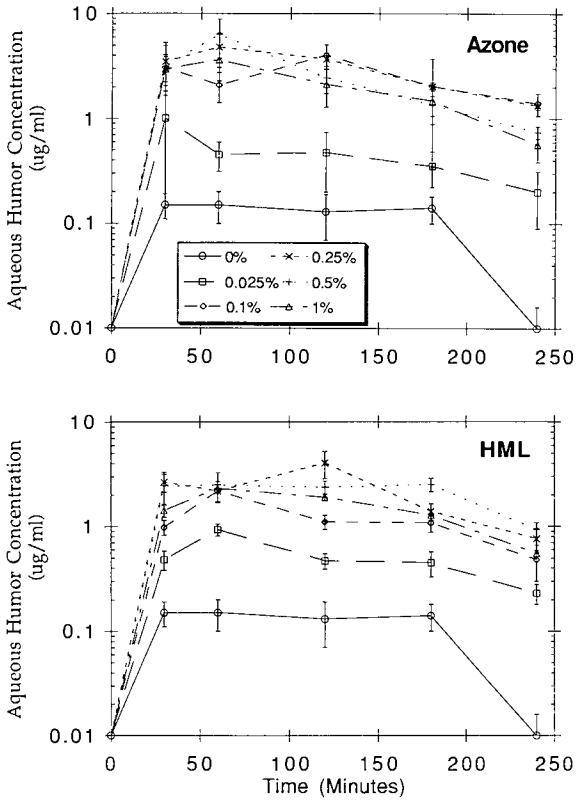

Fig. 2. Concentration–time profiles of cimetidine in aqueous humor when a 1% cimetidine eyedrop containing various concentrations of Azone or hexamethylene lauramide was instilled in rabbit eyes.

magnitude of increase in ocular absorption of cimetidine was observed in the presence of trace levels of the enhancers (e.g., 0.1%) and further increases in the enhancer concentration did not improve the enhancement effect significantly.

Table II. Values of Area Under the Aqueous Humor Concentration–Time Curve After One Eyedrop of 1% Cimetidine to Rabbit Eyes[a]

	AUC (μg · min/ml)	
% enhancer	Azone	HML
0	27 (5)	27 (5)
0.025	105 (22)	118 (16)
0.1	588 (96)	273 (42)
0.25	703 (74)	525 (118)
0.5	629 (94)	506 (76)
1.0	483 (66)	353 (20)

[a] Mean (SD).

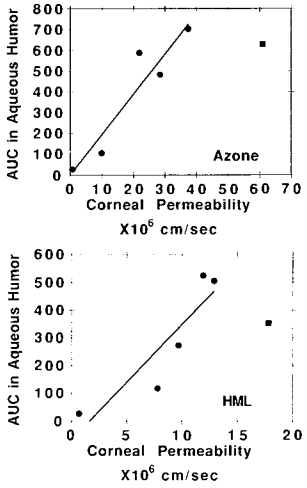

Fig. 3. Relationship between corneal permeability coefficient ($\times 10^6$ cm/sec) (1) and area under the curve in aqueous humor of cimetidine (μg · min/ml) in the presence of different concentrations of Azone (A) and hexamethylene lauramide (B). Open circles were not included in linear regression analysis due to the severe corneal swelling in the presence of high penetration enhancer concentrations.

While Azone seems to be more potent than HML in enhancing ocular absorption of cimetidine at concentrations of 0.1–1%, it is found to be cytotoxic.

Our results indicate that low levels of penetration enhancers can be successfully used to facilitate ocular absorption of poor penetrators, such as cimetidine. However, the effect of penetration enhancer on the well-being of ocular tissues needs to be examined carefully in order to assess the benefits versus the risks of penetration enhancers in ophthalmic dosage forms.

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