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RESEARCH PAPER

Ketoprofen 1-Alkylazacycloalkan-2-one Esters as Dermal Prodrugs: In Vivo and In Vitro Evaluations

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ABSTRACT

Six new 1-alkylazacycloalkan-2-one esters of ketoprofen (**1**–**6**) were synthesized and evaluated as potential dermal prodrugs of ketoprofen. Their lipophilicity by both experimental lipophilicity indices (log k') and calculated ClogP was also determined. In vitro experiments were carried out to evaluate the chemical and enzymatic stability and permeation through excised human skin of these new ketoprofen derivatives. Furthermore, we investigated the in vivo topical anti-inflammatory activity of ester **5**, which showed the best in vitro profile, evaluating the ability of this compound to inhibit methyl nicotinate-induced skin erythema on healthy human volunteers. Esters **1**–**6** showed increased lipophilicity compared with the parent drug (ketoprofen), good stability in phosphate buffer pH 7.4, and were readily hydrolyzed by porcine esterase. Results from in vitro percutaneous absorption studies showed that, among all esters synthesized, only for esters **1** and **5** did a higher cumulative amount of drug penetrate through the skin, compared with that obtained after topical application of ketoprofen. In vivo results showed an interesting delayed and sustained activity of ester **5**, compared with the parent drugs.

Key Words: Dermal prodrug; Skin permeation; Ketoprofen; *N*-Alkyllactams; Controlled release.

INTRODUCTION

Topical nonsteroidal anti-inflammatory drugs (NSAIDs) are widely available for the treatment of acute and chronic painful inflammations of the muscles and joints. Evidence is reported that the local administration of NSAIDs induces less adverse effects than when given orally,[1] and this is probably related to their much lower plasma concentrations when they are topically applied than when they are

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ingested.[2] Furthermore, topical drug efficacy mainly depends on its ability to penetrate the skin to such an extent as to elicit the desired pharmacological activity. Ketoprofen is an interesting NSAID, and it is widely prescribed for patients affected by dermatitis[3] and rheumatic diseases.[4] Because ketoprofen shows unsuitable physicochemical properties, various strategies to aid its skin penetration have been studied. Among these strategies, the prodrug approach represents a well-known tool used to enhance the skin permeability of drugs. The prodrugs are bioreversible, pharmacologically inactive derivatives of a drug molecule that require a chemical or enzymatic transformation to release the active parent in situ.[5] This strategy is an alternative to the use of penetration enhancers extensively employed to increase ketoprofen skin permeability,[6–8] but are often toxic, irritating, or allergenic.[9] Recently, we obtained several dermal prodrugs of some NSAIDs, conjugating them by ester linkage to different penetration enhancers[10–14] as *N*-alkyllactams, *N*-acyllactams, polyoxyethylene glycols, and terpenes. Some of these prodrugs showing good water stability, rapid enzymatic hydrolysis, and increased fluxes through excised human skin can be regarded as interesting NSAIDs dermal prodrugs. In the present study, to evaluate the potentiality of using *N*-alkyllactam as promoieties in ketoprofen dermal prodrug design, a number of 1-alkylazacycloalkan-2-one esters of this drug (**1**–**6**) have been synthesized and characterized to assess their chemical and enzymatic hydrolysis and fluxes through excised human skin. *N*-Alkyllactams were chosen as promoieties because they are regarded as skin penetration enhancers,[15] and lactamic rings are present in some of the most effective skin penetration enhancers like Azone and *N*-methylpyrrolidone. Another objective of the present study, in order to investigate the relationship between in vitro skin permeation data and in vivo topical anti-inflammatory activity, was to evaluate the ability of esters **1**–**6** to inhibit methyl nicotinate (MN)-induced erythema on healthy human volunteers.

MATERIALS AND METHODS

Apparatus

Melting points were taken on a Buchi 510 capillary melting point apparatus and are uncorrected. The infrared (IR) spectra were measured on a Perkin Elmer model 281 spectrometer utilizing potassium bromide disks. ^{1}H-NMR spectra were recorded with a Bruker model WP 80, using $CDCl_3$ as solvent and trimethylsilane as internal standard. Elemental analysis was performed on a Carlo Erba model 1108 elemental analyzer (Carlo Erba, Milan, Italy). The HPLC system consisted of a Waters model 600 pump with a model 486 UV-Vis detector, a Wisp model 712 automatic sample injection module, a Waters C_{18} μ Bondpak, 4.6 mm × 30 cm reversed-phase column, and a NEC APCIV computer.

Chemicals

Ketoprofen was obtained from Sigma Chemical (St. Louis, MO). 2-Pyrrolidinone, δ-valerolactam, and ε-caprolactam were purchased from Aldrich Chemical (Milwaukee, WI). Methanol and water used in the high-performance liquid chromatography (HPLC) procedures were of liquid chromatographic grade (LC) and were bought from Carlo Erba. All other chemicals and solvents were of reagent grade. All 1-(2-hydroxyethyl)azacycloalkan-2-ones were prepared by refluxing each lactam sodium salt with 2-bromoethanol in anhydrous xylene for 5 hr as described in the literature.[16] All 1-(3-chloropropyl)-azacycloalkan-2-ones were prepared by refluxing each lactam sodium salt with *n*-tetrabutylammonium bromide and 3-bromo-1-chloropropano in anhydrous xylene for 24 hr as described in the literature.[17]

Preparation of Ketoprofen Acid 1-Ethyl-azacycloalkan-2-one Esters (1–3)

A mixture of 1-(2-hydroxyethyl)azacycloalkan-2-one (0.15 mol), ketoprofen (0.15 mol), and neutral alumina (10 g) in xylene (50 mL) was refluxed under azeotropic conditions until the stoichiometric amount of water was removed. The alumina was then filtered off and the filtrate was washed with a 5% aqueous solution of sodium carbonate and water, dried over anhydrous sodium sulfate and evaporated in vacuo. The residue obtained was purified through a column of silica gel using ethyl acetate–cyclohexane (80:20); from the evaporation of the eluent phase, we obtained compounds **1** and **2** (solid) and compound **3** (oil) (Fig. 1). The melting points, yields, IR data, and ^{1}H-NMR chemical shifts of esters **1**–**3** are listed in Table 1. Elemental analyses (C, H, N) were within ±0.3% of the theoretical value.

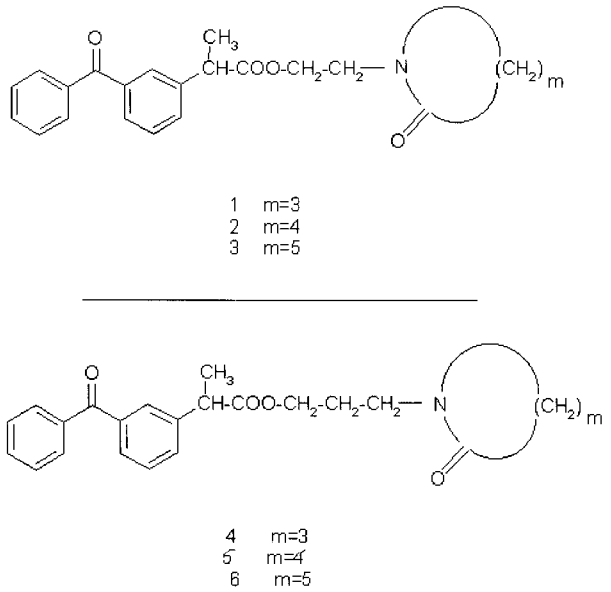

Figure 1. Chemical structure of esters **1–6**.

Preparation of Ketoprofen Acid 1-Propyl-azacycloalkan-2-one Esters (4–6)

Esters IV–VI were prepared using a slight modification of the method reported by Nielsen.[18] A mixture of 1-(3-chloropropyl)azacycloalkan-2-one (0.02 mol), ketoprofen (0.01 mol), potassium iodide (0.001 mol), and triethylamine (0.01 mol) in ethyl acetate (50 mL) was refluxed under azeotropic conditions until the stoichiometric amount of water was removed. Triethylamine chlorohydrate was then filtered off, and the filtrate was first washed with 2 M HCl and then with a 5% aqueous solution of sodium carbonate and water. The solution was dried over anhydrous sodium sulfate for 24 hr and evaporated in vacuo. The residue obtained was purified through a column of silica gel using ethyl acetate–cyclohexane (60:40); from evaporation of the eluent phase, we obtained the compounds IV–VI (oils) (Fig. 1). The melting points, yields, IR data, and ^{1}H-NMR chemical shifts of esters IV–VI are listed in Table 1. Elemental analyses (C, H, N) were within ±0.3% of the theoretical value.

Determination of Chemical and Enzymatic Stability

The hydrolysis rate of ester derivatives (**1–6**) in solution of isotonic phosphate buffer [pH 7.4 ($\mu = 0.5$)] was determined at 32°C after the disappearance of the ester by HPLC analysis. Enzymatic hydrolysis of esters **1–6** was determined as previously described.[11] Briefly, porcine esterase was diluted 1,000-fold with isotonic phosphate buffer before use. Fifty microliters of methanolic ester solution (10^{-4} M) were diluted with 3 mL of isotonic phosphate, thermostated at 37°C, and then 100 µL of the esterase solution was added. Concentration of the ester in solution was monitored by the HPLC method reported herein. Pseudo–first-order constants for chemical and enzymatic hydrolysis were determined from the slopes of linear plots of the logarithm of residual ketoprofen esters against time.

Lipophilicity Indices ($\log k'$) and Calculated Partition Coefficients (ClogP) of Ketoprofen and Esters 1–6

Lipophilicity indices of esters **1–6** were obtained by the isocratic HPLC method measuring compound retention times expressed as $\log k'$.[19] It is well known that the ranking of elution in reversed-phase chromatography represents a relative scale of lipophilicity of analytes. Estimates of lipophilicity of derivatives **1–6** were also obtained considering the theoretically calculated values of $\log P$ (ClogP) (ClogP 4.0 for Windows; Biobyte Corp., Claremont, CA).

HPLC Analysis of Ketoprofen and Esters 1–6

Ketoprofen and its ester derivatives **1–6** were determined by HPLC using a mobile phase consisting of methanol and phosphate buffer (65:35). The chromatograph was run at ambient temperature at a flow rate of 1.4 mL/min. The column effluent was monitored continuously at 254 nm. The retention times are reported in Table 2. The compounds were quantified by measuring the peak areas compared with those of standards chromatographed under the same conditions.

In Vitro Skin Permeation Experiments

Samples of adult human skin (mean age 43 ± 8 years) were obtained from breast reduction operations. Subcutaneous fat was removed, and the skin

Table 1. Yield, melting point, IR, and ^{1}H-NMR data for esters **1**–**6**.

Compound	Yield (%)	Melting point (°C)	IR (KBr cm^{-1})			^{1}H-NMR (δ, ppm)
			$Ph_2C{=}O$	$-CH_2-N-C{=}O$	$-O-C{=}O$	
1	28.4	77–78	1,650	1,675	1,735	4.22 (t, –O–CH_2–CH_2–N<); 3.81 (q, Ph–CH–CH_3); 3.48 (t, –O–CH_2–CH_2–N<); 1.54 (d, Ph–CH–CH_3)
2	26.6	59–60	1,640	1,655	1,735	4.20 (t, –O–CH_2–CH_2–N<); 3.85 (q, Ph–CH–CH_3); 3.57 (t, —O–CH_2–CH_2–N<); 1.58 (d, Ph–CH–CH_3)
3	37.2	Oil	1,645	1,655	1,735	4.21 (t, –O–CH_2–CH_2–N<); 3.80 (q, Ph–CH–CH_3); 3.55 (t, –O–CH_2–CH_2–N<); 1.54 (d, Ph–CH–CH_3)
4	28.5	Oil	1,645	1,680	1,735	4.21 (t, –O–CH_2–CH_2–CH_2–N<); 3.81 (q, Ph–CH–CH_3); 3.39 (t, –O–CH_2–CH_2–CH_2–N<); 2.10–1.60 (m, –O–CH_2–CH_2–CH_2–N<); 1.58 (d, Ph–CH–CH_3)
5	26.4	Oil	1,640	1,660	1,735	4.11 (t, –O–CH_2–CH_2–CH_2–N<); 3.82 (q, Ph–CH–CH_3); 3.41 (t, –O–CH_2–CH_2–CH_2–N<); 2.10–1.60 (m, –O–CH_2–CH_2–CH_2–N<); 1.54 (d, Ph–CH–CH_3)
6	39.3	Oil	1,645	1,655	1,735	4.10 (t, –O–CH_2–CH_2–CH_2–N<); 3.81 (q, Ph–CH–CH_3); 3.52 (t, –O–CH_2–CH_2–CH_2–N<); 2.10–1.60 (m, –O–CH_2–CH_2–CH_2–N<); 1.54 (d, Ph–CH–CH_3)

was immersed in distilled water at 60 ± 1°C for 2 min,[20] then stratum corneum and epidermis (SCE) were peeled off. The SCE membranes were dried in a desiccator at approximately 25% relative humidity and then wrapped in aluminum foil and stored at 4 ± 1°C until used.[21] Dried SCE samples were rehydrated by immersion in distilled water, at room temperature, for 1 hr before being mounted in Franz-type diffusion cells (LGA, Berkeley, CA). The skin surface available for absorption was 0.75 cm^2, and the receptor volume was 4.5 mL. The receiving compartment contained ethanol/water 50:50 to ensure the sink conditions.[22] The receiving solution was stirred and maintained at 35 ± 1°C throughout the experiments. Ketoprofen, and its esters (**1**–**6**) were dissolved in ethanol (5 mg/mL), and 200 μL were placed on the skin surface. The solvent was allowed to evaporate, and the experiments were run for 24 hr. Samples of the receiving solution (50 μL) were withdrawn at 24 hr and analyzed for ketoprofen and its esters (**1**–**6**) content by the HPLC method described herein.

Preparation of Aqueous Gel

Carbomer gels, containing ketoprofen and esters **1**–**6** were prepared by dispersing carbopol 934

Table 2. Molecular weight, retention time (T_r), chemical and enzymatic hydrolyses ($t_{½}$), calculated partition coefficient (ClogP), lipophilic indices ($\log k'$) and cumulative amount penetrating through excised human skin after 24 hr (Q) of ketoprofen and esters **1**–**6**.

Compounds	Molecular weight	$t_{½}$		$C \log P$	$\log k'$	$Q \pm$ SD[a] (μmol·10^2/cm^2)	T_r (min)
		Buffer pH 7.4	Porcine esterase				
Ketoprofen	254.3	—	—	2.8	0.1	12.8 ± 2.1	2.4
1	365.6	8.7 days	2.5 hr	2.9	0.4	24.8 ± 3.7[b]	3.7
2	379.4	8.5 days	2.8 hr	3.5	0.8	11.9 ± 2.9[c]	6.7
3	393.2	9.2 days	2.3 hr	4.1	1.1	8.7 ± 1.7	13.0
4	379.4	16.5 days	4.1 hr	2.5	0.6	5.8 ± 1.6	4.8
5	393.2	18.2 days	4.7 hr	3.0	0.9	37.5 ± 5.6	9.1
6	407.8	17.3 days	5.2 hr	3.6	1.2	11.3 ± 2.4	15.5

[a]Each experiment was run in duplicate on three different donors.
[b]Ester **1** vs. ester **5**: $p < 0.01$.
[c]Ketoprofen vs. ester **2**: $p < 0.05$.

(Carbomer; 1.5% w/w) in distilled water (73–75.5% w/w) with constant stirring. The drugs (8 mmol) or their esters **1**–**6** (8 mmol) were solubilized in ethanol (50% w/w), together with methyl-*p*-hydroxybenzoate (0.1% w/w). The ethanolic solution was added to the carbopol dispersion, and the mixture was then neutralized and made viscous by the addition of triethanolamine (2.0% w/w). The gels were stored at room temperature for 24 hr under air-tight conditions before use.

In Vivo Anti-inflammatory Activity of Esters 1–6 on MN-Induced Erythema

Instrument

The induced MN erythema was monitored by using a reflectance visible spectrophotomer X-Rite model 968, having 0° illumination and a 45° viewing angle, as previously reported.[10] The instrument was calibrated with a supplied white standard traceable to the National Bureau of Standards perfect white diffuser. The spectrophotomer was controlled by a IBM Pentium III 600 computer, which performed all color calculations from the spectral data by means of a menu-driven suite of programs (Spectrostart) supplied with the instrument. Reflectance spectra were obtained over the wavelength range of 400–700 nm using illuminant C and 2° standard observer. From the spectral data obtained, the erythema index (EI) was calculated using the equation:

$$EI = 100\left[\log\frac{1}{R_{560}} + 1.5\left(\log\frac{1}{R_{540}} + \log\frac{1}{R_{580}}\right) - 2\left(\log\frac{1}{R_{510}} + \log\frac{1}{R_{610}}\right)\right]$$

where $1/R$ is the inverse reflectance at a specific wavelength (560, 540, 580, 510, and 610).

Protocol

In vivo experiments were performed on six volunteers of both sexes in the age range of 25–35 years. Volunteers were fully informed of the nature of the study and the procedures involved. The participants did not suffer from any ailment and were not on any medication at the time of the study. They were rested for 15 min before the experiments, and room conditions were set at 22 ± 2°C and 40–50% relative humidity. Eight sites on the ventral surface of each forearm were defined using a circular template (1 cm^2) and demarcated with permanent ink. For each volunteer, two of the eight sites of each forearm were used as a control, applying 50 mg of gel without active compounds and the other six sites were treated with 50 mg of gels containing esters **1**–**6** or their parent drugs. The preparations were spread uniformly on the site by means of a solid-glass rod. The sites were then occluded for 3 hr using Hill Top Chambers (Hill Top Research, Cincinnati, OH).

After the occlusion period, the chambers were removed and the skin surfaces were washed to remove the gel and allowed to dry for 15 min. On each pretreated site, MN aqueous solution (0.5% w/v) was applied at different times after gel removal: immediately ($t = 0$), 3 hr ($t = 3$), and 6 hr ($t = 6$) later. MN was applied on the skin surface for 1 min using a Hill Top Chamber (1 cm^2) whose cotton pad was saturated with 200 μL of MN solution, and the induced erythema was monitored for 100 min. EI baseline values were taken at each designated site before application of gel formulation, and they were subtracted from the EI values obtained after MN application at each time point to obtain ΔEI values. For each site, the area under the response (ΔEI)-time curve (AUC) was computed using the trapezoidal rule.

RESULTS AND DISCUSSION

Chemical and Enzymatic Stability

In Table 2 are reported the chemical stability data in phosphate buffer of esters **1**–**6**. Esters **4**–**6** appear more stable, compared with 1-ethylazacycloalkan-2-one ester derivatives (**1**–**3**), confirming the trend observed in our previous papers[11] for which the ester group-to-ring distance is decisive in determining the stability of esters. Because an essential prerequisite for the successful use of prodrugs is their reconversion into the parent drug within the skin, we assessed the enzymatic cleavage of esters **1**–**6** using porcine liver esterase that are regarded as a valid model for skin esterase enzymatic activity.[23] Esters **1**–**3** were hydrolyzed more readily, compared with **4**–**6** derivatives, but no significant difference in hydrolysis rate was observed as the ring size of *N*-ethyl and *N*-propyl lactams increased ($p > 0.05$).

Lipophilicity

Stratum corneum is generally believed to be the main barrier in drug skin permeation process. Because the horny layer is basically a lipophilic barrier, drug lipophilicity is regarded as one of the key parameters, but not the only one, that controls drug skin permeation. So, one of the main objectives of many dermal prodrug investigations, as reported in literature, is to obtain prodrug with increased lipophilicity, compared with the parent drug. Many authors[24] have outlined that more lipophilic drug derivatives could show better partitioning and solubility into the SC that could result in enhanced skin permeation.

To assess the lipophilicity of esters **1**–**6**, two different parameters were considered: ClogP data from theoretical calculation and $\log k'$ chromatographic indices.

Regarding our synthesized esters (**1**–**6**), we evaluated their lipophilicity by both measured lipophilic indices ($\log k'$) and calculated ClogP: these parameters increase as the size of the azacycloalkanone ring for each series of derivatives increased (see Table 2).

In Vitro Skin Permeation Study

Skin permeation of ketoprofen and esters **1**–**6** has been evaluated through excised human skin (SCE membranes), because other authors reported that the dermis in vitro can act as a significant additional barrier to the permeation of some compounds.[25] In the case of esters **1**–**6** skin permeation experiments, no hydrolysis of the esters during the skin permeation process was observed. Probably the lack of enzymatic hydrolysis that we found in our in vitro permeation studies is because of SCE membranes obtained by means of a thermal separation technique and the large amount of ethanol used in the receptor compartment for ensuring sink conditions. In vitro skin permeability results were expressed as amounts (μmol) of ketoprofen or its esters **1**–**6** permeated through human skin after 24 hr and are reported in Table 2.

As noted, not all esters increase significantly the cumulative amount of parent drug penetrated through the skin. Besides, among these esters, only for **1** and **5**, was there observed a higher cumulative amount of drug penetrated through the skin, compared with that obtained after topical application of the parent drug. Furthermore, no correlation between skin permeated amounts of ketoprofen or esters **1**–**6** and their lipophilic parameters (ClogP and $\log k'$) was observed.

In Vivo Anti-inflammatory Activity

On the basis of the in vitro results, we investigated the topical anti-inflammatory activity of ester **5** that had shown the best in vitro results. To evaluate the in vivo anti-inflammatory activity of this derivative, carbomer gels containing ester **5** (8 mmol/100 g) or

ketoprofen (8 mmol/100 g), were prepared. Different models have been reported in the literature for evaluating topical anti-inflammatory activity of NSAIDs.[26] Among these models, UV-B and MN-induced erythema are the most used in humans.[10,27] Reflectance filter colorimetry has been extensively used[28] for designating the extent of erythema by measuring the skin color surface in terms of CIE (Commission International d'Eclairage) L*a*b* color space parameters, because some authors[29,30] found significant correlation between a^* values and visual grading of skin erythema. Reflectance spectrometry provides skin reflectance spectra, generally in the range of 400–700 nm, from which it is possible to obtain EI values for more accurate and reliable evaluations of skin erythema.[31] From the ΔEI values, calculated at each site and at different times, it was possible to monitor the extent of MN-induced skin erythema and the ability of ester **5** to inhibit this process after their preventive application onto the skin, using carbopol gels as vehicle formulations. In Fig. 2, the typical time courses of MN-induced erythema on skin sites pretreated with ester **5** and with ketoprofen are reported. Plotting ΔEI values vs. time, AUC values were determined, for each subject, by calculating the areas between the response curve and the x axis, and the mean AUC values are reported in Table 3. AUC values were inversely related to tested substance ability to inhibit MN-induced erythema. MN application was effectuated at different times ($t=0$ hr, $t=3$ hr, $t=6$ hr) after active compound removal. As reported in Table 3 at $t=0$ and $t=3$, ketoprofen was more effective than ester **5** in inhibiting induced erythema and only at $t=6$ did this ester show a significantly inhibitory activity. Furthermore, it is possible from AUC values to calculate the percentage of inhibition of erythema induced by MN to outline better the

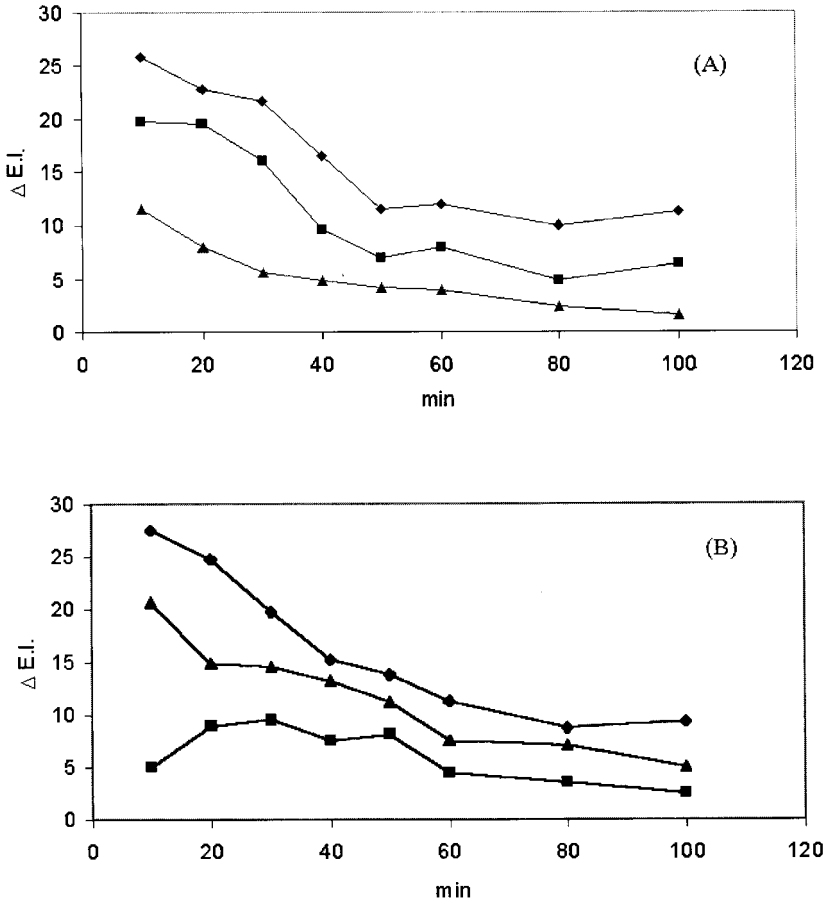

Figure 2. ΔEI values vs. time for one subject. Ketoprofen or ester V was applied for 3 hr, and MN was applied: (A) immediately after their removal ($t=0$) or (B) 6 hr after their removal ($t=6$). Key: (▲) ketoprofen; (■) ester V; (◆) control.

Table 3. AUC_{0-100} values obtained pretreating skin sites with gel-containing ketoprofen or ester **5** and applying MN immediately after gel removal ($t=0$), 3 hr ($t=3$), or 6 hr ($t=6$) later.

	AUC_{0-100}						
	$t=0$		$t=3$		$t=6$		
Subjects	Ketoprofen	Ester **5**	Ketoprofen	Ester **5**	Ketoprofen	Ester **5**	Control
A	428.3	916.1	461.2	563.4	921.3	475.2	1226.1
B	419.1	885.4	523.9	821.1	1121.1	519.3	1141.5
C	444.6	730.2	538.6	696.3	865.4	396.4	1197.3
D	431.5	947.6	626.4	754.5	1224.6	618.1	1256.5
E	584.2	1319.3	495.2	721.2	918.5	485.7	1049.9
F	396.8	728.6	594.8	698.4	894.7	464.2	1022.3
Mean	450.7	921.2	540.2	709.1	990.9	493.1	1148.9
±SD	67.3	216.3	61.5	85.1	146.0	73.3	95.7

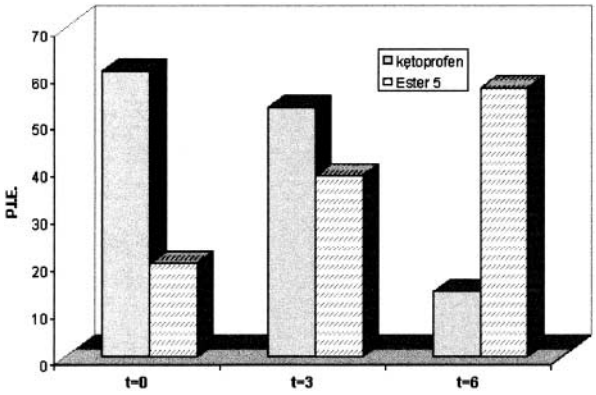

Figure 3. Percentage inhibitions of erythema induced by MN of ester 5 and ketoprofen.

obtained results, where $AUC_{(C)}$ is the area under the response-time curve on the vehicle-treated site (control), and $AUC_{(T)}$ is the area under the response-time curve on the drug-treated site. The percentage of inhibition of erythema values are shown in Fig. 3. As noted, ketoprofen percentage inhibition was maximal when MN was applied immediately after gel removal, although it notably decreased at $t=6$.

In conclusion, on the basis of the results obtained in this work, 1-alkylazacycloalkan-2-one esters appear to be suitable promoieties for ketoprofen dermal prodrug design. So, esters **1** and **5** showed the main requirements needed for dermal prodrugs, such as chemical stability, enzymatic lability, and increased in vitro skin permeation. Furthermore, the in vivo topical anti-inflammatory activities of ester **5**, using MN-induced erythema in human volunteers as inflammation models, pointed out an interesting delayed and sustained activity compared with ketoprofen.

$$\text{Inhibition}\,(\%) = \frac{AUC_{(C)} - AUC_{(T)}}{AUC_{(C)}} \times 100$$

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