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In vitro evaluation of a series of Azone analogs as dermal penetration enhancers. I

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Summary

The influence of a series of Azone analogs on the percutaneous penetration of a lipophilic model drug (hydrocortisone-21-acetate) across hairless mouse skin has been investigated. Methods of synthesis of these novel compounds are also described. Permeability studies utilized vertical non-occluded Franz cells at 37°C and propylene glycol as the vehicle for the drug. Enhancers were applied one h prior to drug treatment in the same vehicle. Three enhancers were applied at their maximum saturation solubilities in propylene glycol, the rest of the compounds at 0.4 M. Enhancement ratios were calculated for flux, 24 h diffusion cell receptor concentrations, and full-thickness skin total steroid contents. All enhancers were found to increase permeation parameters to a greater or lesser extent over control. A few compounds were found to be more effective than Azone in increasing these parameters; particularly skin retention of the model drug.

Introduction

The principal barrier to drug permeation in skin is the stratum corneum. In recent years there have been many attempts to increase the flux of drugs through this layer using compounds known as penetration enhancers (Barry, 1987; Hirvonen et al., 1991). Enhancers interact reversibly with stratum corneum constituents to disrupt the highly ordered structure and hence facilitate drug diffusion.

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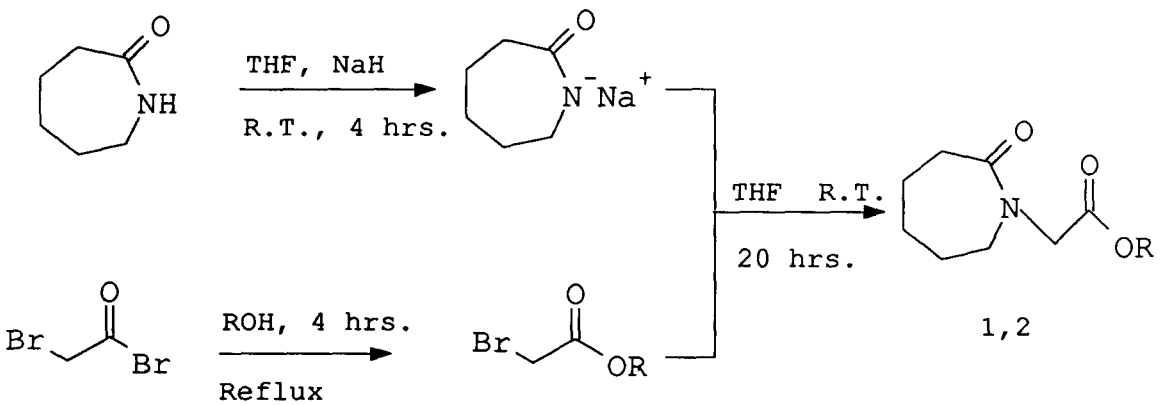
Many established penetration enhancers are synthetic chemicals which are not yet approved by regulatory authorities for use with drugs. *N*-Dodecylazacycloheptan-2-one (Azone) has been shown to be a potent enhancer of transdermal transport of antibiotics, glucocorticoids, and 5-fluorouracil (Stoughton, 1982a) as well as peptides (Bannerjee and Ritschel, 1989; Boddé et al., 1989). It is likely that Azone and alkyl-azones act in a similar way by fluidizing the lipid barrier (Barry, 1987; Bouwstra, 1992). It has also been reported that Azone can be applied undiluted to skin with no significant discomfort even at a concentration above 50% (Stoughton, 1982b). Many authors are at present reporting the en-

hancement effects of various Azone analogs (Okamoto et al., 1991; Hoogstraate et al., 1991).

Propylene glycol has been shown, in combination with various enhancers (e.g. oleic acid and Azone), to increase the penetration of the drug compared with each vehicle alone (Cooper, 1984; Wooton et al., 1985). Azone in combination with propylene glycol enhanced the permeation of hydrocortisone (Barry and Bennett, 1987). Propylene glycol is a vehicle commonly used in pharmaceutical formulations. Other enhancers which have recently been tested are *N,N*-dimethylamides (C_3–C_{10} alkyl) which appear to be more active at lower concentrations than *N,N*-dimethylformamide and *N,N*-dimethylacetamide (Irwin et al., 1990). Many enhancers have been investigated in the literature in addition to the ones mentioned, such as alcohols (ethanol), surface-active agents, and water (Walters, 1990; Zatz, 1991). Most of these increase transdermal drug fluxes but few of these studies reported drug concentrations within the skin itself, since the purpose of the enhancers was to increase the flux of drugs such as levonorgestrel or nitroglycerin into the systemic circulation. This study attempts to determine whether or not an enhancer can be designed which would increase concentrations of a model drug within the skin, this being the target drug delivery site, rather than the systemic circulation.

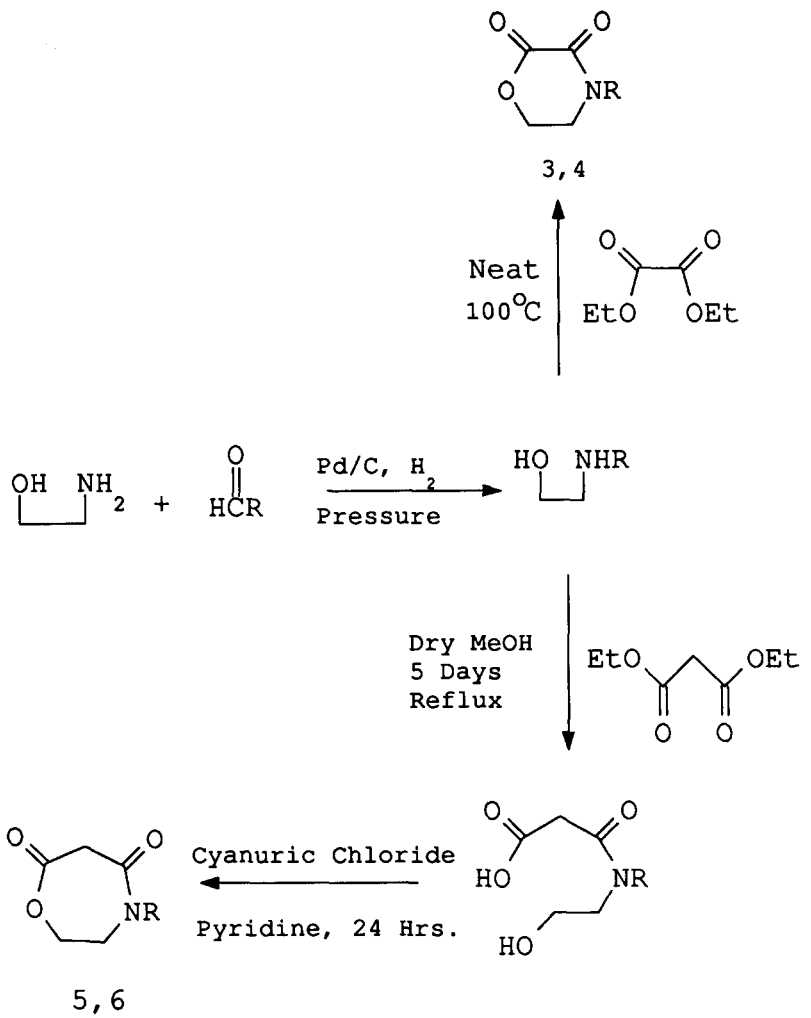
The structures of the various Azone analogs synthesized as part of this investigation are presented in Schemes 1–4. The permeation enhancement of each was tested against hydrocortisone-21-acetate in hairless mouse skin in vitro.

A variety of synthetic strategies were employed. Caprolactam was reacted with sodium hydride in dry tetrahydrofuran, then *N*-alkylated with dodecyl or tetradecyl bromoacetate or 1-bromododecane to yield compounds **1, 2** and Azone, or acylated with decanoyl, lauroyl or myristoyl chloride to yield compounds **10–12** (Schemes 1 and 4). In contrast, there were several possibilities for preparing the *N*-alkylmorpholine-2,3-diones (**3, 4**). The oldest procedures (Tam, 1986; Murihashi, 1988) involving carbonylation



Compound	R
1	n-$C_{12}H_{25}$
2	n-$C_{14}H_{29}$

Scheme 1.

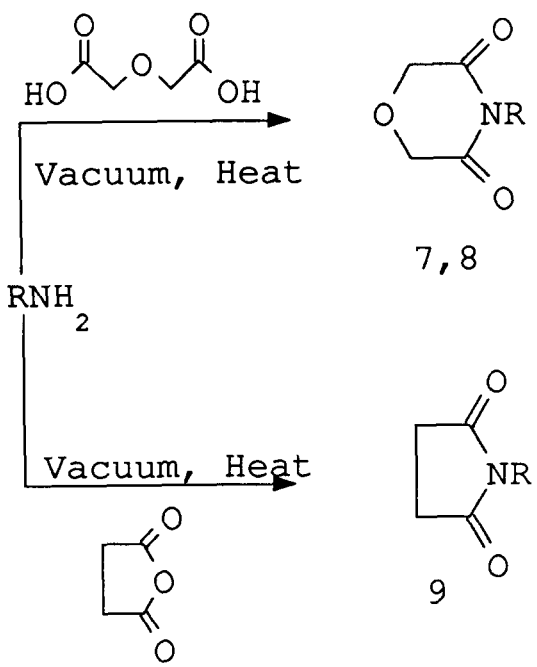


Compound	R
3	n-$C_{12}H_{25}$
4	n-$C_{14}H_{29}$
5	n-$C_{10}H_{21}$
6	n-$C_{12}H_{25}$

Scheme 2.

under heat and pressure were rejected as cumbersome. Addition of ethyl oxalyl chloride to the intermediate *N*-alkylethanolamine was attempted but *N*- and *O*-acylation predominated. The compounds were finally prepared by heating diethyl oxalate with the *N*-alkylethanolamine (Scheme 2)

(Skinner et al., 1980). While the 1,4-oxazepine-5,7-dione ring is available via the reaction of carbon suboxide and ethanolamine (Bonsignore et al., 1987), this reaction is of limited versatility. In our case this would have required *N*-alkylation of a potentially sensitive ring. Other reported methods, such as direct coupling of malonyl dichloride and the *N*-alkylethanolamine results in low yields (10–15%) and numerous by-products. We found that forming the intermediate ε-hydroxyacid followed by lactonization with cyanuric chloride/pyridine (Scheme 2) (Brown et al., 1988) gives the highest yields of **5** and **6**. Finally, the *N*-alkylmorpholine-3,5-diones (**7**, **8**) and the *N*-dodecylsuccinimide (**9**) were prepared by heating the amine with diglycollic acid or succinic anhydride (Scheme 3) (Sido, 1921).



Compound	R
7	n-$C_{12}H_{25}$
8	n-$C_{14}H_{29}$
9	n-$C_{12}H_{25}$

Scheme 3.

Materials and Methods

Materials

All chemicals were purchased from Aldrich Chemical Co. in the highest available purity, except hydrocortisone-21-acetate, hydrocortisone, polyoxyethylene 20 cetyl ether and propylene glycol which were obtained from Sigma Chemical Co. Baxter Diagnostics, Inc. supplied reagent grade solvents, except for methanol and acetonitrile which were HPLC grade. All were used as received except tetrahydrofuran which was distilled over metallic sodium immediately prior to use and methanol which was dried over magnesium according to the procedure of Lund and Bjerrum (Furniss et al., 1978) for the preparation of **5** and **6**.

Male hairless mice strain SKHI (hr/hr), 8 weeks old, were obtained from Charles River Laboratories, Inc., Wilmington, MA.

Methods

Synthetic characterization

Infrared spectra were recorded on a Beckman Acculab 4 spectrophotometer either neat or using the potassium bromide technique and ^{1}H-NMR spectra were obtained on a Bruker AM 300 NMR spectrometer. The recorded spectra agreed with the compound's structure in all cases. Elemental analyses were conducted by Atlantic Microlabs, Atlanta, GA, and were within ±0.4% of the theoretical value in all cases.

Permeability experiments

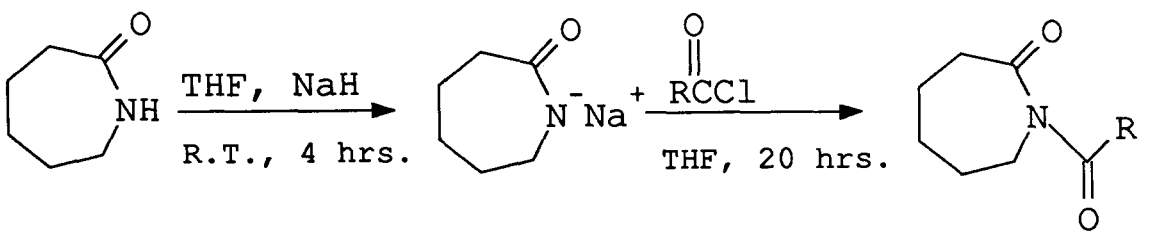
In vitro diffusion cell experiments The model drug hydrocortisone-21-acetate was used to characterize the barrier properties of the skin. Full-thickness skins were placed in all-glass, modified Franz diffusion cells (Vangard International, Neptune, NJ) at 37 ± 0.5°C. The diffusional area of the skin was 3.14 cm^2. The surface tempera-

ture of the skins was at 32 ± 0.5°C. The receptor phase was continuously stirred at 600 rpm and consisted of phosphate buffer (isotonic, pH 7.2) with 0.1% v/v of 36% aqueous formaldehyde added as a preservative (Sloan et al., 1991). Hydrocortisone acetate has an aqueous solubility of 1 mg/100 ml, and hydrocortisone 0.28 mg/ml (Windholtz, 1983). In order to maintain sink conditions and increase the solubility in the receptor phase, 0.5% w/v polyoxyethylene 20 cetyl ether was added to the receptor phase (Chien, 1982; Loftsson et al., 1989).

Dorsal skin samples were placed in diffusion cells and allowed to equilibrate for 1.5 h. At this time the enhancer solution (0.4 M) in 5 ml of propylene glycol was spread evenly on each skin. This pre-treatment method avoided enhancer effects on the thermodynamic activity of the model drug. Several of the enhancers were solids at 32 ± 0.5°C. The solubilities of these compounds in propylene glycol were investigated, and if 0.4 M enhancer was not soluble in the vehicle, the maximum solubility (in M) was determined and results are presented in Table 2. All experiments were performed with shaking at 32 ± 0.5°C in 0.5 ml propylene glycol. 1 h later, 0.03 M hydrocortisone acetate suspension in 500 μl propylene glycol was placed on each skin. Saturation solubility of hydrocortisone acetate in propylene glycol has been reported to be 0.003 M (Davis et al., 1991).

Samples (100 μl) were withdrawn from the receptor phase at pre-determined times for 24 h. The receptor was replenished with 100 μl of fresh buffer after each sample was taken. Analysis of each subsequent sample was corrected for all previous samples that had been removed for analysis. Each experiment was repeated at least five times.

Skin retention studies After 24 h, skins were removed and immersed repeatedly in 100 ml methanol for 5 s. Solubility of hydrocortisone acetate in methanol was 3.9 mg/ml (Windholtz, 1983). The time of contact with methanol was minimized in order that only surface steroid was removed from each skin. The skins were then dried for 10 min, weighed, cut up with scissors, and 4 ml of methanol was added. Average skin weight was 0.1069 ± 0.0954 g ($n = 50$). Homogenization was performed using a Kinematic GmbH (Switzerland) tissue homogenizer at a medium



10-12

Compound	R
10	$n\text{-}C_9H_{19}$
11	$n\text{-}C_{11}H_{23}$
12	$n\text{-}C_{13}H_{27}$

Scheme 4.

setting. Samples were filtered to remove debris, centrifuged if required and frozen at −80°C prior to analysis by HPLC. This method, a modified version of that reported by Sasaki et al., (1991), has been described previously (Michniak et al., 1993).

Recovery experiments were performed on eight individual diffusion cells. The methanol washes were sampled (n = 3) and the steroid content was analyzed by HPLC. This represented total donor concentration remaining after 24 h. Skin steroid content (total) in μg per individual skin weight, together with total corrected receptor concentration (μg/12 ml) after 24 h was added. These results yielded > 95% recovery of the original donor concentration of hydrocortisone acetate.

HPLC analysis of samples

Analysis of samples was performed on a Waters 6000A solvent delivery system Model U6K injector (injection volume 50 μl), Waters differential UV detector Model ALC 202, an Alltech C18 Versapack reverse-phase column (4.1 mm × 30 cm; 10 μm) at ambient temperature, and a Hewlett Packard 3390A integrator.

Hydrocortisone and hydrocortisone acetate were detected at 254 nm and were eluted from the column using 4 parts acetonitrile : 6 parts water mobile phase at 1 ml min^{-1}. The enhancer compounds were found to have a λ_{max} in the range of 200–245 nm and were found not to interfere with the steroid HPLC assay.

The receptor phase was found to contain only hydrocortisone (metabolite), however, skin samples contained mainly the acetate with lesser amounts of hydrocortisone. It is possible that hydrolysis was taking place in the receptor phase over the 24 h without the presence of esterase activity (Tauber, 1987). The retention time for hydrocortisone was 5.6 min and that for hydrocortisone-21-acetate was 13.2 min. Quantification was made using external standards.

Data analysis

Permeation profiles for hydrocortisone acetate were constucted by plotting total amount in mol per l penetrating in time t (h). The x-intercept of the extrapolated linear region of the profile (i.e., steady state) gave lag time, and maximum flux was calculated from the slope. Sink conditions

TABLE 1

Effect of enhancers on flux, 24 h receptor concentrations and steroid skin contents of hydrocortisone-21-acetate

Group no.	Enhancer in PG [a]	L [b] (h)	Flux [e] (μM cm^2 h^{-1})	ER_{flux}	Q_{24} [c,e] (μM)	$ER_{Q_{24}}$	SC (HCA) [d,e] (μg g^{-1})	SC (HC) [d,e] (μg g^{-1})	ER_{SC} (HC and HCA)
1	none (n = 8)	1.16 ± 0.32	0.045 ± 0.016	1.00	0.751 ± 0.250	1.00	285.2 ± 21.6	ND	1.0
2	Azone (n = 5)	0.73 ± 0.09	0.878 ± 0.251	19.51	28.760 ± 4.624	38.30	410.6 ± 34.4	9.9 ± 2.5	1.5
3	**1** (n = 5)	1.34 ± 0.08	0.051 ± 0.015	1.13	2.905 ± 1.092	3.87	435.7 ± 37.9	13.5 ± 1.9	1.6
4	**5** (n = 5)	0.85 ± 0.02	2.023 ± 0.561	44.96	57.471 ± 6.425	76.89	1467.9 ± 95.0	27.6 ± 3.4	5.2
5	**6** (n = 5)	1.24 ± 0.14	0.570 ± 0.114	12.67	22.475 ± 4.299	29.93	324.2 ± 23.9	20.1 ± 2.3	1.2
6	**7** (n = 5)	0.75 ± 0.08	0.459 ± 0.126	10.20	9.635 ± 1.626	12.83	337.7 ± 18.2	6.6 ± 1.2	1.2
7	**8** (n = 5)	0.62 ± 0.05	0.633 ± 0.159	14.07	10.874 ± 2.091	14.48	360.8 ± 19.5	8.6 ± 2.1	1.3
8	**9** (n = 5)	0.66 ± 0.02	0.846 ± 0.169	18.80	11.150 ± 3.152	14.85	874.4 ± 29.2	11.3 ± 3.6	3.1
9	**10** (n = 5)	0.59 ± 0.05	0.551 ± 0.106	12.24	11.231 ± 3.065	14.96	508.4 ± 18.3	7.3 ± 2.1	1.8
10	**11** (n = 5)	1.06 ± 0.20	0.647 ± 0.129	14.38	13.780 ± 3.595	18.35	650.3 ± 28.4	6.1 ± 0.9	2.3
11	**12** (n = 5)	0.25 ± 0.09	0.900 ± 0.154	20.00	16.059 ± 3.989	21.38	675.3 ± 29.6	9.8 ± 0.8	2.4

[a] PG, propylene glycol.

[b] L, lag time.

[c] Q_{24}, receptor concentration in diffusion cell after 24 h.

[d] SC, skin content of hydrocortisone-21-acetate (HCA) + hydrocortisone (metabolite) (HC).

[e] Statistically significant differences ($p < 0.05$, Student's t-test) were established between the following group numbers: [1, 3 < 2, 4–11], [2 < 4] for flux, between [1 < 2–11], [2 < 4], [9–11 < 2, 4, 5] for Q_{24}, and between [1 < 2–4, 7–11], [2 < 4] for skin steroid contents.

were assumed to exist, i.e., high donor concentration compared to low receptor during the times flux was measured. Receptor concentrations were also recorded after 24 h although at this time steady-state conditions were not maintained and were seen as plateauing of the flux vs time graphs. Results are presented as enhancement ratios ER where:

ER = (permeation parameter after enhancer treatment)

/(permeation parameter from control)

and permeation parameter denotes the flux, receptor concentration after 24 h or total steroid skin content. All statistical analyses were performed using Student's *t*-test.

Results and Discussion

The data obtained were plotted as cumulative amount of drug (M) detected in the receptor with time. These graphs showed an initial lag phase followed by a linear phase which was consistent with steady state Fickean diffusion. Regression of the linear portion of the graph gave lag time and flux values presented in Table 1. Skin samples contained a mixture of hydrocortisone acetate and metabolite hydrocortisone expressed as total steroid in $\mu g\ g^{-1}$ of hydrated skin. Average skin weights were 0.1152 ± 0.0491 g ($n = 20$). The receptor phase however, contained only hydrocortisone. The steroid was probably metabolized by esterases in the skin and to an extent without esterase activity (Tauber and Rost, 1987). Elucidation of these pathways was beyond the scope of this paper and hence, only total steroid concentrations were reported. All compounds tested showed some degree of enhancement which was reflected to a greater extent in flux and 24 h receptor concentrations than in skin contents (Table 1). Compound **5** showed the highest enhancement ratios. Flux increased from 0.045 ± 0.016 (control) to 2.023 ± 0.561 $\mu M\ cm^{-2}\ h^{-1}$, Q_{24} values from control 0.751 ± 0.250 to 57.741 ± 6.425 μM, and skin steroid contents from control 285.2 ± 21.6 to 1495.5 ± 98.4 $\mu g\ g^{-1}$. With such high concentrations at 24 h, sink conditions were not maintained as was shown by significant plateauing of the cumulative receptor concentration vs time plots. These values were also much higher than the aqueous solubility of hydrocortisone would allow (0.28 mg/ml) which substantiates the use of a solubilizer in the receptor compartment (Chien, 1982; Windholtz, 1983; Loftsson et al., 1989). Surfactant compounds have previously been reported to affect skin permeability to some extent (Ponec et al., 1990) and therefore polyoxyethylene 20 cetyl ether was also included in all control experiments. All enhancers listed in Table 1 were found to be miscible or soluble at a concentration of 0.4 M in propylene glycol at a 32 ± 0.5°C skin surface temperature. However, compounds **2–4** were solids and were not soluble in propylene glycol at such high concentrations. The saturation solubility for these compounds was determined and suspensions of these compounds were applied to the mouse skin. Compound **2**, even at saturation solubility of 0.136 M (approx. 5%), enhanced skin steroid content by 7.6-fold. Most enhancers decreased the lag times,

TABLE 2

Effect of enhancers 2–4 on flux, 24 h receptor concentrations and steroid skin contents of hydrocortisone-21-acetate

Group no.	Enhancer in PG	C_{ss} (M)	L (h)	Flux [a] ($\mu M\ cm^2\ h^{-1}$)	ER_{flux}	Q_{24} [a] (μM)	$ER_{Q_{24}}$	SC (HCA) [a] ($\mu g\ g^{-1}$)	SC (HC) [a] ($\mu g\ g^{-1}$)	ER_{SC} (HC and HCA)
12	**2** ($n = 5$)	0.136	0.22 ± 0.08	0.088 ± 0.014	1.96	2.411 ± 1.046	3.25	2167.4 ± 85.9	ND	7.6
13	**3** ($n = 5$)	0.113	1.02 ± 0.20	0.319 ± 0.154	7.09	14.782 ± 1.995	19.68	729.4 ± 25.7	3.7 ± 0.7	2.6
14	**4** ($n = 5$)	0.051	0.48 ± 0.15	0.234 ± 0.199	5.20	4.362 ± 1.486	5.81	296.1 ± 21.3	2.1 ± 0.6	1.1

C_{SS}: saturation solubility of enhancer in propylene glycol. Other symbols are as in Table 1. [a] Statistical differences ($p < 0.05$, Student's *t*-test) were established between the following group numbers: 1 < 13, 14], [13, 14 < 4] for flux, and between [1 < 12–14], [12–14 < 2, 4] for Q_{24}, and between [1 < 12, 13], [4 < 12], [2 < 12, 13] for skin steroid contents.

for example, Azone and compounds **4, 5** and **7–12**.

However, it must be stressed that such values are subject to error particularly if obtained from linear regression extrapolation to the time axis. Compounds **10–12** have previously been reported by Nelson Research (Minaskanian et al., 1986) and were included for comparative purposes.

Azone was found to be an effective enhancer for increasing flux values and 24 h receptor concentrations (ER values: 19.51 and 38.30, repectively) rather than skin retention. However, compound **5** showed significantly higher ER values (44.96 and 76.89, respectively) and compounds **1–3, 5** and **9–12** all showed higher ER values for steroid skin content compared with Azone. Since all these compounds had lower flux ER values than Azone, with the exception of **5** and **12**, we conclude that it is possible to increase skin drug retention while only moderately increasing flux. This effect may be enhancer concentration- and vehicle-dependent, as well as being influenced by the chemical structure of the enhancer. This question will have to be answered in future investigations.

Enhancers **1** and **2** contained an ester linkage that was probably prone to hydrolysis by esterases which are found at relatively high concentrations in hairless mouse skin (Ghosh et al., 1990). Nevertheless, these compounds did show activity, particularly **2**, which at a saturation solubility of 0.136 M increased skin steroid content by 7.6-fold over control. The presence of seven-membered rings also seemed important for higher activity (compounds **2, 5, 10–12** and Azone). It must be stressed that such high enhancement ratios as observed in this study will not be seen with human skin which is known to be far less permeable (Barry, 1983; Roberts et al., 1990). However, the authors feel that compounds **2–9** are excellent candidates for further testing as potential novel transdermal penetration enhancers.

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