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A mechanistic investigation of the in vitro human skin permeation enhancing effect of Azone®

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Abstract

In order to clarify the mechanism of the enhancing action of Laurocapram (Azone) on the skin permeation of drugs, the following experiments were conducted. Partitioning of an homologous series of alkyl anilines into *n*-octanol and isopropyl myristate (IPM) in the absence and presence of Azone (1%, 5%, and 10%, w/w) was determined. The addition of 1% and 5% of enhancer to *n*-octanol and 1% of enhancer to IPM slightly increased the partition coefficients of the alkyl anilines assayed. This effect was more pronounced for 10% Azone in both cases. The percutaneous penetration-enhancing effect of Azone for similar concentrations through the human skin was investigated using 5-fluorouracil as a hydrophilic model permeant, four compounds selected from the alkyl anilines (aniline, 4-ethylaniline, 4-*n*-butylaniline and 4-*n*-pentylaniline) with a wide range of lipophilicity values (log PC_{Oct} from -0.87 to 4.2 where PC_{Oct} is the octanol/water partition coefficient) and two compounds which belong to the phenyl alkanols series, 2-phenylethanol and 4-phenylbutanol with lipophilicity values of log PC_{Oct} of 1.18 and 2.34, respectively. The enhancer effect of Azone depended predominantly on two parameters, the concentration of the enhancer used and the lipophilicity of the compound assayed. When the concentration of the Azone employed was 1% (w/w), it only acted on the compounds with a lipophilicity value of less than log PC_{Oct} of 1 but if the enhancer concentration was increased to 5% (w/w) this lipophilicity threshold increased to a log PC_{Oct} value of 2.69. Therefore the number of drugs whose penetration may be increased by the use of Azone will be greater at the higher concentration. The less the lipophilicity value of the penetrant, the greater the enhancer effect observed. In the case of 10% Azone the penetration of the compounds tested could be also increased for 5-FU, aniline, 4-ethylaniline, 2-phenylethanol and 4-phenylbutanol, but for this last compound there is a reduction in the degree of penetration if it is compared with the permeability coefficients obtained with 5% of Azone. Finally, the Azone cannot enhance the skin permeability for the highest lipophilic compounds (log $PC_{Oct} \geq 3$).

Keywords: Azone; Enhancer concentration; Partition coefficient; Human skin; Permeability

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1. Introduction

Azone (1-dodecylazacycloheptan-2-one) is a non-irritant compound (Wiechers et al., 1988) that can be used for enhancement of the skin permeation of drugs (Stoughton and McClure, 1983; Touitou, 1986; Morimoto et al., 1986). Recently, permeation studies have suggested that Azone acts on the structured lipids of the stratum corneum and enhances the intercellular penetration of drugs by means of the induction of increased fluidity in this region (Hadgraft, 1991; Okamoto et al., 1991; Sugibayashi et al., 1992). However, the complete picture of the penetration-enhancing mechanism of Azone is still unclear.

There are few reports which have dealt systematically with the interaction between the physicochemical properties of the penetrant and the effect of the percutaneous penetration enhancer (Watkinson et al., 1993). The present work was carried out to investigate the effects of Azone at different concentrations on the permeation of a series of compounds through human skin in-vitro. 5-Fluorouracil was used as a hydrophilic model permeant along with four compounds (aniline, ethylaniline, butylaniline and pentylaniline) with a wide range of lipophilicity values (log PC_{Oct} from 0.14 to 4.2) selected from the alkyl aniline homologous series as models of basic compounds. Two compounds belonging to the phenylalkanols series (2-phenylethanol and 4-phenylbutanol with a log PC_{Oct} values of 1.18 and 2.34, respectively) were also examined as models of non-ionic penetrants. In addition, the mechanism of the penetration-enhancing effect of Azone is discussed in relation to the possibility that the enhancer could modify the penetration/lipophilicity correlation (parabolic model) established previously (Díez-Sales et al., 1991b) without the presence of enhancer.

2. Materials and methods

5-Fluorouracil (Sigma Chemical Company, Lot 49F3476), aniline (Merck), 4-methylaniline (Merck), 4-ethylaniline (Merck), 4-*n*-propylaniline (Janssen Chimica Co.), 4-*n*-butylaniline (Aldrich Chemical Co.), 4-*n*-pentylaniline (Aldrich Chemical Co) and 4-n-hexylaniline (Aldrich Chemical Co), 2-phenylethanol (Aldrich Chemical Co.) and 4-phenylbutanol (Aldrich Chemical Co.) were used as received. The compounds were prepared as saturated solutions buffered to pH 6.2. Azone was a gift from Whitby Research (USA). All HPLC solvents were of Hypersolv grade.

All analysis was conducted by HPLC. The system used consisted of an LDC ConstaMetric 3500 pump a Spark-Holland Marathon autosampler (50 μl loop) and an LDC SpectraMonitor 5000 Photo Diode Array UV detector set at 254 nm. Data collection and integration were conducted using the LCtalk software package. Analytical Spherisorb S5 ODS2 columns (150/4.6 mm) equipped with 5 mm Guardpack RCSS-C18 precolumns were employed. The mobile phases consisted of mixtures of acetonitrile and phosphate buffer solution (pH 6.2) in variable proportions, depending on the lipophilicities of the tested solutes and were delivered at a flow rate of 1 ml/min at ambient temperature. A calibration graph was determined for each compound and excellent linearity between peak area and concentration was observed for every compound over the entire range of concentrations assayed.

2.1. Partitioning experiments

The effect of Azone on the partition coefficients of permeants was examined using *n*-octanol and isopropyl myristate (IPM) as the organic phases with 0%, 1%, 5% and 10% of the enhancer dissolved in the solvent. Organic phases without enhancer and with the presence of added enhancer were presaturated with the buffer solution. Aqueous buffer phases (pH 6.2) were presaturated with the appropriate organic phase. Aliquots of the aqueous phases were put into flasks with the organic phase. Flasks were maintained at 25°C and stirred for 2 days. Samples of aqueous phase were removed using a glass syringe, centrifuged twice to remove any residual organic phase and then suitably diluted for analysis by HPLC. The partition coefficients (PC) were calculated according to Eq. (1) below (Irwin and Smith, 1991):

Table 1
Partition coefficients of compounds between *n*-octanol, isopropyl myristate (+ Azone) and buffer solution (pH 6.2) at 25°C. Means of three to four experiments are listed; values given in parentheses are standard deviations

Compound	*n*-Octanol[a]				Isopropyl myristate[b]		
	None	1	5	10	None	1	10
5-Fluorouracil	0.144[c]	n.d.	n.d.	0.170[c]	n.d.	n.d.	n.d.
Aniline	6.70 (0.83)	9.02 (0.14)	8.91 (0.34)	11.5 (0.18)	6.74 (0.15)	7.19 (0.23)	10.6 (0.54)
Methylaniline	25.5 (0.39)	30.1 (1.02)	26.1 (0.20)	35.8 (0.73)	17.6 (0.13)	17.9 (0.26)	25.1 (0.57)
Ethylaniline	81.4 (3.2)	98.9 (2.0)	98.5 (1.5)	105 (1.7)	54.6 (1.1)	56.9 (0.4)	75.3 (1.2)
Propylaniline	388 (2.8)	467.7 (19)	482 (9.9)	589 (12)	250 (10.5)	266 (1.8)	405 (13.5)
Butylaniline	948 (14.7)	972 (17)	1063 (8.13)	1189 (7.7)	1662 (58)	1780 (44)	2692 (36)
Pentylaniline	6239 (152)	6633 (612)	6978 (292)	7424 (137)	2573 (89)	2855 (87)	3398 (181)
Hexylaniline	15266 (874)	15605 (268)	15901 (633)	16125 (918)	11757 (313)	14727 (1748)	15190 (222)
Phenylethanol	15.13[d]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Phenylbutanol	173.78[d]	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

[a] None, 1, 5, and 10% (w/w) of Azone was added to *n*-octanol.[b] None, 1 and 10% (w/w) of Azone in isopropyl myristate.[c] From Okamoto et al., 1991.[d] Calculated using MedChem software (Daylight Systems). Not determined (n.d.).

$$PC = \frac{C_1 \cdot V - C_2 \cdot V}{C_1 \cdot V - C_2 \cdot V + C_2} \quad (1)$$

where C_1 and C_2 are the initial and final concentration of solute in the aqueous phase, respectively. The phase volume ratio (V) is equal to V_W/V_O. When equal volumes of aqueous (V_W) and organic phases (V_O) are used $V = 1$ and Eq. (1) reduces to Eq. (2):

$$PC = \frac{C_1 - C_2}{C_1} \quad (2)$$

2.2. Permeation experiments

All permeation experiments were performed on Caucasian abdominal skin samples (female aged 38–48 years), obtained from cosmetic surgical corrections. Excess fatty and connective tissues were removed and the samples stored in a freezer at −20°C for less than 2 months. Epidermal membranes were prepared by a heat-separation technique. The skin was immersed in water at 60°C for 45 s after which the epidermis was carefully separated from the underlying dermis using forceps.

All diffusion studies were performed using epidermal membranes from human skin mounted in Franz type diffusion cells with the stratum corneum towards the donor compartment which contained 2 ml of saturated drug solution. The skin samples were placed in the diffusion cells with the desired section centred on the cell to give an effective area available for diffusion of 0.78 cm^2. The receiver compartment capacity was approximately 6 ml and the temperature was maintained at 37 ± 1°C by immersion of the cells in a water bath. The dermal side of the skin was continuously washed with saline solution buffered to pH 7.4 and stirred by a rotating teflon-coated magnet placed inside the cell. The receptor solution was added with polysorbate 80 at a clearly supramicellar concentration (1%, w/w) in order to provide a micellar reservoir and, consequently, sink conditions were completely fulfilled (Díez-Sales et al., 1991a). The receptor solution was in close contact with the epidermal sheet during the whole experiment. The epidermal membranes

Table 2
The permeability coefficients (K_p) with standard deviations (in parentheses) of the mean of all compounds tested in human epidermal membranes before and after treatment with Azone

Compound	Molecular weight	Permeability coefficients ($K_p \cdot 10^3$, cm/h)				
		Control	Ethanol	1%	5%	10%
5-Fluorouracil	130.1	0.601 (0.09)	0.685 (0.09)	3.52 (0.41)	6.51 (0.13)	10.75 (0.28)
Aniline	93.1	61.05 (5.74)	57.86 (5.74)	72.37 (6.15)	86.00 (9.34)	99.10 (8.76)
4-Ethylaniline	121.5	286.75 (14.92)	271.98 (34.48)	311.64 (15.35)	362.14 (19.43)	461.96(24.49)
4-*n*-Butylaniline	149.24	411.16 (11.33)	411.65 (31.15)	420.00 (46.48)	476.42 (38.39)	388.08 (41.98)
4-*n*-Pentylaniline	163.3	226.30 (10.39)	219.63 (14.94)	227.10 (11.10)	214.90 (17.16)	217.01 (19.96)
2-Phenylethanol	122	30.25 (5.12)	36.25 (2.53)	32.15 (4.01)	115.02 (9.14)	147.50 (16.74)
4-Phenylbutanol	150	86.17 (7.25)	92.14 (4.74)	93.22 (5.37)	164.70 (13.06)	106.95 (8.94)

were pretreated with either 0.1 ml of ethanol or the equivalent amount of an ethanolic solution of Azone (1%, 5% or 10%, w/w) overnight. At zero time, a 2 ml aliquot of the saturated solution of the compound at pH 6.2 was then applied to each donor cell. Samples of 0.2 ml were taken from the receptor compartment every 60 min over a time frame of 36 h. The volume withdrawn was always replaced with an equal volume of fresh receptor solution. Throughout the experiments, the donor cell was capped with a silicon stopper to prevent the donor solution from evaporating.

To examine the effect of the 12 h pretreatment of skin with ethanol and the ethanolic solutions of Azone, drug penetration through non-pretreated skin was determined (control experiments). A drug saturated donor solution was applied to the excised epidermal membrane without any pretreatment.

Eq. (3) was used to fit experimental data (Scheuplein, 1967):

$$Q_{(t)} = A \cdot P \cdot h \cdot C \cdot \left[D \cdot \frac{t}{h^2} - \frac{1}{6} - \frac{2}{\pi^2} \sum_{n=1}^{\infty} \frac{(-1)^2}{n^2} \cdot Exp\left(\frac{-D \cdot n^2 \cdot \pi^2 \cdot t}{h^2} \right) \right] \quad (3)$$

where $Q_{(t)}$ is the quantity which passes through the membrane and reaches the receptor solution at a given time, t, A represents the actual diffusional surface area (0.78 cm^2), P the partition coefficient of the permeant between the membrane and the donor vehicle, h the membrane thickness, D is the diffusion coefficient of the permeant in the membrane and C is the concentration (here its solubility) of the permeant in the donor solution. The terms $P \cdot h$ and D/h^2 were replaced in Eq. (3) by P_1 and P_2, respectively, and calculated through fitting the theoretical equation to individual in-vitro permeation data sets using a computerized non-linear least squares method. The permeability coefficients, K_p ($= P_1 \cdot P_2$), were calculated and used as representative permeation parameters.

The enhancement ratio (ER) was calculated, as by Williams and Barry (1991) (Eq. (4)):

$$ER = \frac{K_p \text{ after application of penetration enhancer}}{K_p \text{ before application of penetration enhancer}} \quad (4)$$

Values reported are mean ratios from a minimum of four replicates.

2.3. Fitting of models to data

Correlations between permeability coefficients (K_p) and lipophilicity indexes for homologous series of xenobiotics were used as the most suitable source of information about the passive permeation mechanisms and the role of hydrophilic-lipophilic alternate barriers in penetration. A classical parabolic equation approach was used:

$$\log K_p = a \cdot \log (P)^2 + b \cdot \log (P) + c \quad (5)$$

Table 3
Statistical significance of the differences between K_p values of each compound tested under different conditions, control (I), ethanol (II), 1% Azone (III), 5% Azone (IV) and 10% Azone (V)

Compound	Statistical differences ($P <$)				
	I–II	I–III	III–IV	III–V	IV–V
5-Fluorouracil	NS	***	***	***	*
Aniline	NS	*	*	*	*
4-Ethylaniline	NS	NS	**	***	***
4-*n*-Butylaniline	NS	NS	NS	NS	NS
4-*n*-Pentylaniline	NS	NS	NS	NS	NS
2-Phenylethanol	NS	NS	***	***	*
4-Phenylbutanol	NS	NS	***	*	***

NS, not significantly different.*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

The fitting operations were developed using an IBM-PC computer with the aid of a non-linear least-squares regression program (Yamaoka et al., 1981). In this equation, *a*, *b* and *c* are constants which can be experimentally calculated depending on the technique.

2.4. Statistical analysis

A logarithmic transformation of permeability coefficients was used in order to obtain homogeneous variability. Homogeneity was confirmed by Bartlett's test. One- and two-way ANOVA was used previously to Peritz's test (Harper, 1984).

Akaike information criterion, AIC (Akaike, 1976) as well as the correlation coefficient between experimental and model-predicted K_p values were used to assess the goodness of the fit.

3. Results and discussion

In order to clarify the mechanism of action of Azone on the skin permeation of drugs the enhancing effects for several concentrations of enhancer were investigated using compounds with a wide range of lipophilicity values. Permeability coefficients and partitioning coefficients into *n*-octanol and IPM in the presence and absence of enhancer were obtained.

For the homologous series of alkylanilines the partition coefficients between the two different organic solvents and water are listed in Table 1. The addition of 1% and 5% Azone (w/w) in *n*-octanol (PC_{Oct}) and 1% Azone in IPM (PC_{IPM}) increased the partition coefficients slightly. This effect was more apparent for 10% Azone in both solvents.

Table 2 shows the mean permeability coefficients of all compounds tested in accordance with the conditions described previously. As can be observed in Table 3 there are no significant differences between the permeability values for untreated membranes (control) and those determined when the membrane had been previously treated with ethanol. Consequently, the pretreatment of the membrane with ethanol does not produce an increase in penetration. However, after treatment of the membranes with 1% of an ethanolic solution of Azone only two compounds, 5-fluorouracil (ER = 5.9) and aniline (ER = 1.3) show a significant increase in the penetration value (Table 3). It is interesting to note that both compounds have a lipophilicity value of $PC_{Oct} < 10$ (Table 1).

When pretreatment of the membranes was carried out using a 5% ethanolic solution of Azone all the compounds tested showed a significant increase in their permeability coefficients (Table 3), except the 4-*n*-pentylaniline. The greater the enhancer effect observed, the less the lipophilicity value of the penetrant. It is therefore possible that the Azone (5%) produces some morphological change in the stratum corneum lipid structure that

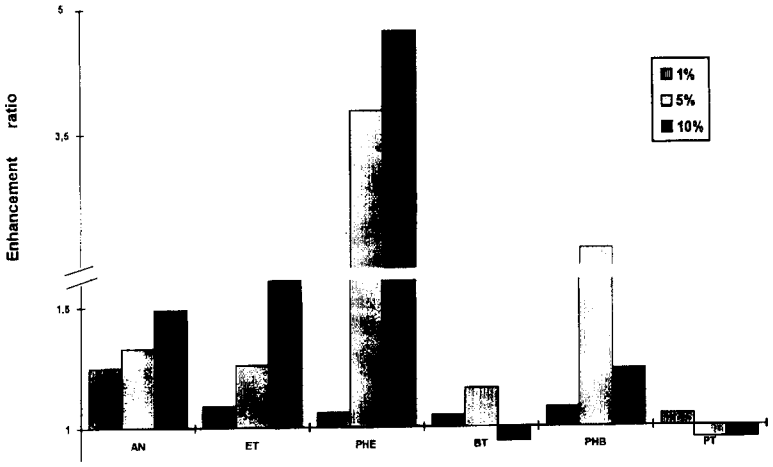

Fig. 1. The penetration enhancing activity of Azone for different concentrations (1%, 5%, 10%, w/w) expressed as enhancement ratios. AN, aniline; ET, 4-ethylaniline; PHE, 2-phenylethanol; BT, 4-*n*-butylaniline; PHB, 4-phenylbutanol; PT, 4-*n*-pentylaniline.

is more drastic than when the treatment of the membranes was carried out a the lower concentration of enhancer (1%). In the case of the 4-*n*-pentylaniline, for which the enhancement ratio was practically equal to 1 (Fig. 1), probably the absence of enhancer effect could be a consequence of its high lipophilicity ($\log PC_{Oct} = 4.2$).

Treatment of the skin membranes with the 10% ethanolic solution of Azone also produces an enhancement in the permeability values for 5-FU, aniline, 4-ethylaniline, 2-phenylethanol and 4-phenylbutanol, but for the 4-*n*-butylaniline and 4-*n*-pentylaniline there are not significant differences (Table 3). However it should be noted that although 4-phenylbutanol shows an increase in its permeability value, in regard to the control, the enhancement ratio (ER = 1.3) is less than what was observed when skin treatment was carried out with 5% of enhancer (ER = 1.9). These results show that as the compounds are of moderate and high lipophilicity, i.e. rapidly penetrating molecules, the use of a high concentration of Azone can lead to a reduction or even an elimination of the enhancer effect. This fact could be explained if it is taken into account that the Azone, in addition to the fluidificant action on the skin lipids, exerts an effect on the hydration of the stratum corneum (Sugibayashi et al., 1992), so that as the concentration of Azone was increased the water content should increase. This condition makes the penetration of hydrophilic compounds easier, nevertheless for the most lipophilic compounds the partition into the 'hydrated' stratum corneum is made difficult and, consequently, the penetration is reduced.

The results (Table 2) for the permeability coefficients obtained with different concentrations of Azone could indicate that the penetration values for the compounds tested, which have a lipophilicity values ($\log PC_{Oct}$) from 0.14 (5-FU) to 1.9 (4-ethylaniline) are a function of the concentration of enhancer employed. Therefore, as a greater concentration of Azone is used more penetration enhancement may occur.

Furthermore, Fig. 1 shows that there are differences in penetration rates of 2-phenylethanol and 4-ethylaniline in spite of the similarity in their molecular weights (Table 2). Similar behaviour is exhibited by 4-phenylbutanol and 4-*n*-butylaniline when Azone is used in 5% concentration. There is a greater increase in penetration of the compound with the lower lipohilicity value.

Perhaps the most widespread procedure for attempting to clarify the mechanisms of percutaneous absorption is to establish correlations between representative penetration parameters (e.g. permeability coefficients) and measurements of lipophilicity or other related physico-chemical

Table 4
Equation parameters describing the correlations established between permeability coefficients (K_p, cm/h) obtained through epidermis from human skin without pretreatment (control) and with pretreatment of the membrane for Azone (5%, 10%, w/w), and lipophilicity for the compounds tested

Equation parameters	4-Alkyl chain lengths (N)			Partition coefficients n-octanol/water		
	Control	5%	10%	Control	5%	10%
a	−0.097(0.021)	−0.011(0.003)	−0.088(0.009)	−0.239(0.009)	−0.274(0.007)	−0.262(0.099)
b	0.651(0.142)	0.715(0.223)	0.504(0.060)	1.309(0.049)	1.469(0.036)	1.337(0.517)
c	−1.438(0.442)	−1.410(0.334)	−0.996(0.084)	−2.166(0.065)	−2.256(0.047)	−2.004(0.644)
AIC	−22.05	−17.65	−24.61	−14.46	−15.29	−4.17
r	0.995	0.990	0.997	0.995	0.998	0.990

characteristics. Several in vitro kinetic models for studying percutaneous penetration have been proposed. In accordance with previous 'in vitro' permeation studies for the alkylanilines through human skin (Díez-Sales et al., 1991b) the probabilistic model was found to be the most adequate in this case. This model (i.e. a parabolic equation) implies that an optimal chain length value for penetration exists. In this work, the values of permeability coefficients through human skin obtained for alkylanilines under different conditions of pretreatment with Azone (5 and 10% ethanolic solutions) and without pretreatment of the membrane (control) were correlated with the number of methylene groups in the 4-n-aliphatic chain by means of the same equation. Equation parameters for the control and the two concentrations of Azone assayed are shown in Table 4. Plots relating permeability coefficients (K_p) and alkyl chain length by means of a parabolic equation (Eq. (5)) are reproduced in Fig. 2. Note that the maxim of the curves appear at 3,3 (control), 3,2 (Azone, 5%) and 2,8 (Azone, 10%) carbon atoms, respectively. Log PC_{Oct} values equal 2.7, 2.69 and 2.54, respectively. Thus Azone appears to increase the penetration rates of compounds that have values of lipophilicity lower than the optimum lipophilicity value in the absence of enhancer. Thus, for compounds that have a lipophilicity value greater than this optimum, Azone does not produce an increase in the penetration rate and its use may even induce a decrease.

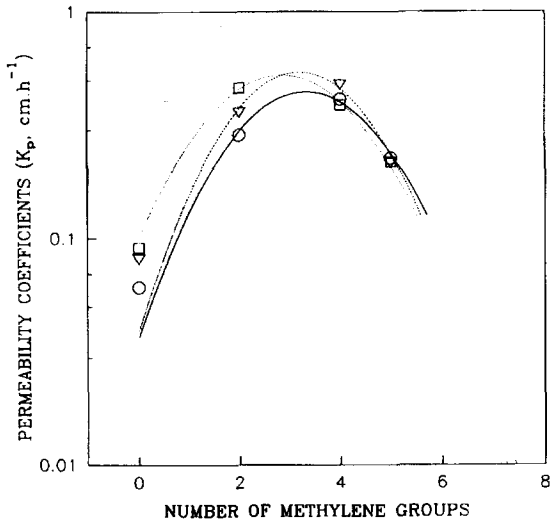

Fig. 2. Plots relating permeability coefficients for alkylanilines obtained through epidermal membranes without pretreatment (○) and with 5% (▽) and 10% (□) of Azone and alkyl chain length in accordance with the parabolic model.

In conclusion, Azone can be used with a wide spectrum of compounds but the enhancer effect depends mainly on two parameters, the concentrations of the enhancer used and the lipophilicity value of the penetrant in question. When the concentration of the Azone used was 1% (w/w) it only acted on compounds with a lipophilicity value of log $PC_{Oct} \leq 1$ (5-FU and aniline). When the concentration was 5% (w/w) the lipophilicity limit for the enhancement increased to log$PC_{Oct} = 2.69$. In this case, therefore, the number of drugs that will be usefully enhanced will be greater. Finally, if the membrane was treated with a 10% Azone solution increase in penetration rates can be also obtained depending of the lipophilicity permeant. For lipophilic compounds

the penetration rate can even show a reduction in the enhancement ratio when compared with that at 5% Azone (4-phenylbutanol). However, Azone might not enhance the skin permeability of compounds with a high lipophilicity (log $PC_{Oct} \geq 3$) independently of the concentration of enhancer employed.

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