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In vitro permeation and irritation of benzoyl peroxide-containing products

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Abstract

Two in vitro models to measure release of benzoyl peroxide (BPO) from formulations were presented. Franz-type diffusion cells and acetonitrile/water receptor mediums were used in both models. The barrier in one model was silicone sheeting and in the other was cultured human epidermis. The similarity in release profiles, rates of permeation and diffusion coefficients obtained provided enough evidence to demonstrate that the two models were comparable. An in vitro model to study the irritation of formulations containing BPO was also presented. The model used cell viability as a measure of irritation. Tissue viability was assessed using the MTT test after 5, 19 and 27 h exposure. The effective time where 75% of the keratinocytes remain viable in the skin model (ET_{75}) was used as a measure to compare formulations. ET_{75} decreased as the concentration of BPO increased in the formulations. Values of ET_{75} were lower in vanishing formulations than in tinted formulations with the same level of BPO. Cell viability was linearly related to the rate of release of BPO. The higher the release rate, the lesser was the time needed to reach the ET_{75}. This indicated that the amount of BPO delivered from the vehicle was the primary cause of irritation.

Keywords: Irritation; Permeation; Benzoyl peroxide; Silastic; MTT; Sodium fluorescein

1. Introduction

Benzoyl peroxide (BPO) is the most used active ingredient in all acne products due to its efficacy and availability without prescription. The efficacy of BPO stems from its bactericidal activity against *Propionibacterium acnes* and from its keratolytic effect (Billow, 1993). BPO is superior to antibiotics because the bacteria do not develop resistance to it, and it is preferred over keratolytic agents due to its bactericidal effect. However, a drawback to treatment with BPO-containing products is possible skin irritation to the individual. The degree of irritation is believed to be related to the amount of BPO present in the product (Fulton and Bradley, 1974).

Both the in vitro and in vivo permeation of BPO have been studied extensively by various investigators (Nacht et al., 1981; Yeung et al., 1983; Mena et al., 1994). However, only a few studies compared the permeation of BPO to irritation. In one study (Wester et al., 1991), the authors studied the permeation and irritation of free

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and entrapped BPO from various formulations. The permeation was studied in vitro in human skin and in vivo in monkeys; whereas, the irritation was studied in rabbits and in human beings. Although the information obtained from such a study could be very useful, the experimental work involved was time consuming and too expensive to be performed on a routine basis.

In this study, quick and reproducible in vitro approaches for measuring both irritation and permeation are presented. This will enable scientists to screen various formulations without having to resort to expensive animal or clinical testing. The permeation of BPO was studied in both silicone sheeting and cultured multilayered human epidermis, whereas, the irritation was only studied in the cultured tissue. The results obtained from the two studies were analyzed for comparative purposes.

2. Materials and methods

2.1. Materials

The following were used: Acetonitrile (Burdick and Jackson, Muskegon, MI); Benzoyl peroxide (Aldrich Chemical Company, Milwaukee, WI); Sodium fluorescein (Sigma Chemical Company, Saint Louis, MO); Hydroxy ethylcellulose (Aqualon Company, Wilmington, DE); Water (Burdick and Jackson, Muskegon, MI); MTT (3-[4,5-Dimethyl thiazol 2-yl]-2,5 diphenyltetrazoline bromide) toxicology kit (MatTek Corp., Ashland, MA); High purity sodium lauryl sulfate, NF (Albright and Wilson, Richmond, VA); Benzoyl peroxide formulated as either a 2.5%, 5.0%, 10% vanishing or tinted gels of proprietary composition with BPO freely dispersed (Table 1). Blank vehicles were also prepared.

2.2. Permeation Studies

2.2.1. Permeation Studies with Silastic® Membranes

Franz-type diffusion cells with a 15-mm diameter orifice and a total volume of 7 ml were used. These cells were modified to enable autosampling with a Hanson Microette system. The membrane used was made of non-porous, non-reinforced medical grade silicone sheeting with a thickness of 0.005″ and was supplied by Dow Corning (Midland, Michigan). The receptor phase consisted of a 50/50 (v/v) mixture of water/acetonitrile. During the experiment, the deaerated receptor media was stirred with a magnetic bar at 600 R.P.M. and its temperature was maintained at 32°C.

The synthetic membrane was cut into discs of 35 mm in diameter to fit on top of the diffusion cells. The membrane was soaked in the receptor phase for 30 min, dried and a Teflon® disc with a 15-mm hole in the center was placed over it. The sample was applied and spread evenly across the hole with a Teflon® squeegee. The membrane/disc assembly was placed on the opening of the diffusion cell and was then covered and clamped tightly. The autosampler was programmed to withdraw 1.8-ml samples and replace them with fresh media at: 0.5, 1, 2, 4, 6, 8, 10 and 12 h. These samples were placed into capped HPLC vials that were later transferred to the HPLC for analysis.

2.2.2. Permeation Studies with EpiDerm™

The diffusion cells described earlier were used in this set of experiments as well. The receptor phase consisted of a 60/40 (v/v) mixture of water/acetonitrile which was deaerated before use and maintained at 32°C throughout the experiment. The tissue (EpiDerm™ EPI-606, MatTek Corporation, Ashland, MA) used consisted of normal, human-derived epidermal keratinocytes which

Table 1
Formulations Used in the Study

Gel Formulation		Formula
BPO Concentration % (w/w)	Formula Type	Code
0	Vanishing gel	A
0	Tinted gel	B
2.5	Vanishing gel	C
2.5	Tinted gel	D
5	Vanishing gel	E
5	Tinted gel	F
10	Vanishing gel	G
10	Tinted gel	H

had been cultured to form a multilayered differentiated model of the human epidermis. The tissue contained organized basal, spinous, granular and cornified layers analogous to those found in vivo.

The tissue sample was removed from the cell culture insert using a scalpel and was placed on top of the diffusion cells. The permeation experiment was performed as denoted above with the exception that the samples were withdrawn at the following time intervals: 0.5, 1, 2, 4, 6, 8, 10, 12, 16, 20 and 24 h.

Typically, when cultured skin is used in permeation experiments the receptor phase is usually an isotonic solution containing nutrients and other additives that will keep the tissue alive throughout the experiment (Yang and Kreuger, 1992; Slivka et al., 1993). In some cases saline is used, but saline will only maintain the cell viability for a short period of time. In this study, the use of saline or another water-based receptor medium was impossible due to the extremely low solubility of BPO in such mediums. Instead, a water/organic receptor phase was used with the assumption that the barrier function of the skin would not be affected to a great extent if the skin was not viable. In fact, cadaver skin has the highest barrier function among available membranes (Dick and Scott, 1992; Morimoto et al., 1992; Houk and Guy, 1988). Nevertheless, in order to quantify the possible damage caused by the organic solvents on the barrier function of the tissue, a test recommended by the supplier was performed.

In this test, three discs of tissue from the same lot were clamped in the diffusion cells and exposed to the receptor phase consisting of a 60/40 (v/v) mixture of water/acetonitrile for 6, 12 and 24 h, respectively. After exposure to the solvent, the three discs were removed from the diffusion cells, washed thoroughly with saline and their permeation for sodium fluorescein (SF) was compared to a control. The permeation was monitored for 24 h during which 1.8-ml samples were withdrawn periodically from the receptor phase and analyzed for SF spectrophotometrically. Franz-type diffusion cells were used and the receptor phase consisted of saline maintained at 32°C. The results obtained were very encouraging in that the two pieces of tissue exposed to the solvent for 6 and 12 h didn't show a notable change in their barrier function when compared to the control. However, the test specimen exposed to the solvent for 24 h showed a minor change in its barrier function. These results will be discussed in more detail in the following section.

2.3. Analysis of Samples

2.3.1. HPLC Analysis of benzoyl peroxide

BPO analysis was conducted using a completely automated Spectra-Physics HPLC equipped with a UV detector. A 15-cm C18 reversed phase column with an internal diameter of 4.6 mm was used for the separation. The UV detector was set at 248 nm. BPO was eluted after 3.5 mins with a mobile phase consisting of a 60/40 (v/v) mixture of acetonitrile/water at a flow rate of 1.8 ml/min. Under these conditions, the CV was always less than 5% for 20-μl injections.

2.3.2. Spectrophotometric Analysis of sodium fluorescein

The analysis of SF was conducted with a Hewlett Packard 8452A diode array spectrophotometer. The absorbance at 493 nm was measured and the concentration of SF was calculated using a standard curve.

2.4. In vitro dermal irritancy studies

The tissue (EpiDerm™ EPI-100, MatTek Corporation, Ashland, MA) was handled as instructed in the product handling sheet. Briefly, tissue samples were placed in the assay medium provided. The tissue samples were stored for 1 h in a humidified 37°C, 5% CO_2 incubator prior to being treated with test materials. The assay medium was replaced and 100 μL of each test formula was added to the tissue in triplicate. Untreated tissues and 1% sodium lauryl sulfate (100 μL) treated tissue served as negative and positive controls, respectively. The test material was washed off with phosphate buffered saline pH 7.2. The viability of the tissue was assessed using the MTT toxicology kit after 5, 19 and 27 h exposure. Briefly, tissue samples were incubated with 300 μL MTT for 3 h in a humidified 37°C,

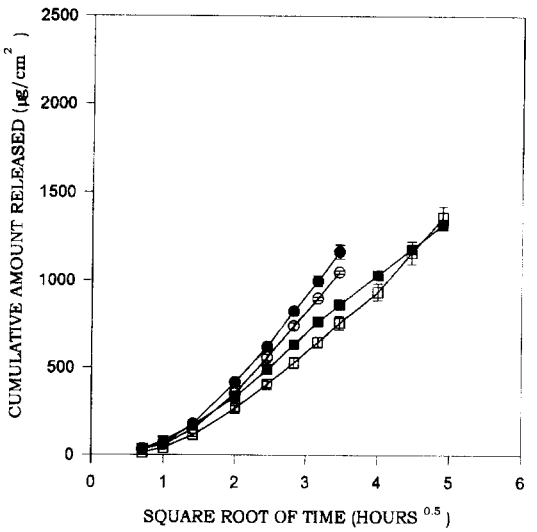

Fig. 1. Effect of membrane type on the in vitro permeation of benzoyl peroxide from formulations containing 2.5% active. Formulation C/Silastic (○); Formulation C/Tissue (□); Formulation D/Silastic (●); Formulation D/Tissue (■).

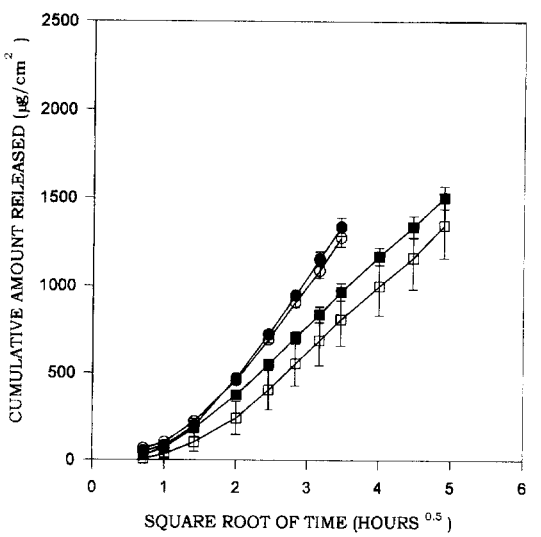

Fig. 2. Effect of membrane type on the in vitro permeation of benzoyl peroxide from formulations containing 5% active. Formulation E/Silastic (○); Formulation E/Tissue (□); Formulation F/Silastic (•); Formulation F/Tissue (■).

5% CO_2 incubator. Following the incubation, the tissue samples were placed in 2.0 ml of MTT extraction solution at room temperature. The optical density of the MTT extraction solution of each sample was read at 570 nm after 2 h using a Thermomax microplate reader (Molecular Devices, Menlo Park, California). Percent viability was calculated as follows:

$$\%\ Viability = \frac{OD_{570nm}(Sample)}{OD_{570nm}(Untreated)} \times 100$$

3. Results and discussion

3.1. In vitro Permeation

The data was processed in a Lotus 1-2-3 spreadsheet and graphs of the cumulative amount of BPO released as a function of the square root of time are shown in Figs. 1–3. In all graphs, a linear relationship with the square root of time was obtained after approximately a 3-h lag time ($r^2 > 0.99$ in most cases). The cumulative amount released increased with an increase in concentration of active in the formula. These results confirm earlier findings by Yeung et al. (Yeung et al., 1983). The release profiles of the vanishing and tinted formulations containing the same amount of BPO were very similar in all cases.

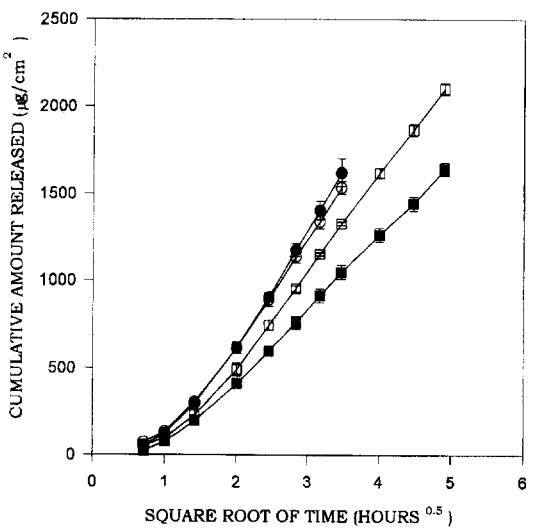

Fig. 3. Effect of membrane type on the in vitro permeation of benzoyl peroxide from formulations containing 10% active. Formulation G/Silastic (○); Formulation G/Tissue (□); Formulation H/Silastic (●); Formulation H / Tissue (■).

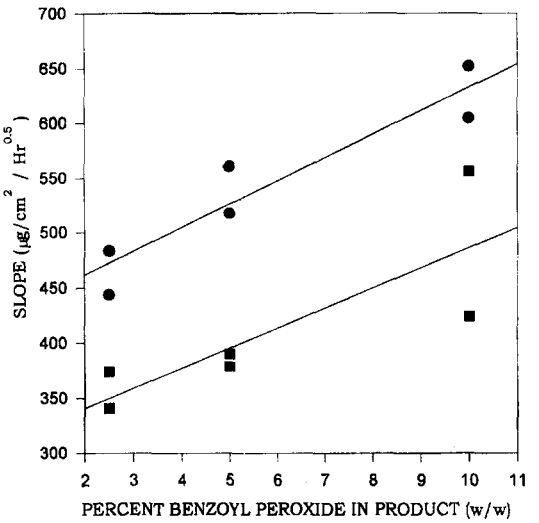

Fig. 4. Relationship between the rate of release of benzoyl peroxide and its concentration in the product. Silastic (●); Tissue (■).

Slopes of the linear portion of the release profiles were calculated. These slopes represented the rate of release of BPO from different formulations. Fig. 4 shows the slopes plotted versus concentration of active in the product for the data obtained from the two barriers. The two linear relationships obtained indicated that the rate of release of BPO was a function of its concentration in the formula and that the rate increased linearly as the amount of BPO in the formula increased. Previously published data (Yeung et al., 1983) have shown similar findings. The regression lines obtained for the two membranes were parallel, with the one obtained for the tissue being lower. This parallelism showed the similarity in trend obtained with the two membranes.

Diffusion coefficients displayed in Table 2 were calculated from the following equation:

$$Q = (2ADCst)^{1/2}$$

Q represents the amount released per unit area in $\mu g/cm^2$; A is the total amount of drug in $\mu g/cm^3$; D is the diffusion coefficient in cm^2/s; Cs is the saturation concentration of drug within the matrix in $\mu g/cm^3$ and t is time (Higuchi, 1961). The value of Cs (0.155 $\mu g/ml$) used in the calculations was obtained from the literature (Chellquist and Gorman, 1992). The diffusion coefficients calculated were found to be concentration dependent and seemed to decrease with an increase in the amount of BPO in the formula. The values of the diffusion coefficients obtained from the experiments using silicone sheeting were slightly higher than the ones obtained when the tissue was used. The difference in diffusion coefficients between the two membranes was expected to be larger. The reason for this expectation is that the permeability coefficient of the tissue used is only slightly lower than cadaver skin which is one or two orders of magnitude less permeable than silicone sheeting (Houk and Guy, 1988; MatTek Corporation Ltd.). It was evident that a change in the permeability of the skin had occurred. This change could not be attributed solely to the effect of solvent since the effect did not change the permeability to a great extent in the fluorescein experiment. Instead, it was suggested that BPO might have damaged the tissue and caused a change in its permeability which would have resulted in higher diffusion coefficients.

In order to verify this possibility, the permeation of SF through skin exposed to formulation G was monitored using Franz-type diffusion cells and saline at 32°C as a receptor phase. In this test, the product was applied over two separate discs of tissue from the same lot placed on top of the diffusion cells. After application, the cells were clamped. The two discs were exposed to the product for 6 and 12 h, respectively. After exposure to the product, the discs were removed from the diffusion cells, washed thoroughly with saline and their permeation for SF was compared to a control (tissue exposed to saline for 12 h). The permeation was monitored for 24 h during which 1.8-ml samples were withdrawn periodically from the receptor phase and analyzed for SF spectrophotometrically. The data obtained were processed in a Lotus 1-2-3 spreadsheet and the cumulative amount of SF permeating through the skin was plotted as a function of time as shown in Fig. 5. As seen from the graph, exposing the tissue to formulation G affected its permeability much more than exposing it to the receptor medium. This finding could explain the high diffusion coefficients obtained when the tissue was used. It

Table 2
Calculated diffusion coefficients

Formulation	Silicone sheeting		Tissue	
	Diffusion Coefficient (cm^2/s)	Standard Deviation	Diffusion Coefficient (cm^2/s)	Standard Deviation
C	7.07×10^{-3}	1.09×10^{-4}	5.03×10^{-3}	5.14×10^{-4}
D	8.39×10^{-3}	7.08×10^{-4}	4.15×10^{-3}	7.99×10^{-5}
E	4.81×10^{-3}	5.48×10^{-4}	2.59×10^{-3}	4.26×10^{-4}
F	5.65×10^{-3}	5.01×10^{-4}	2.74×10^{-3}	2.60×10^{-4}
G	3.82×10^{-3}	3.97×10^{-4}	2.78×10^{-3}	2.54×10^{-4}
H	3.28×10^{-3}	1.65×10^{-4}	1.61×10^{-3}	5.37×10^{-4}

was also observed that the effect of the receptor medium on the permeability of the tissue was negligible; thus, the use of small levels of organic solvents in the receptor phase could be acceptable in this system.

3.2. In vitro irritation

The time response curves for all formulations tested were constructed by plotting cell viability versus dosing time on a semi-log scale (Figs. 6 and 7). Cell viability of the untreated tissue cultures remained unchanged during the experiment. Tissue treated with 1% sodium lauryl sulfate had a cell viability of 14–25%. Formulations A–H caused a decrease in cell viability with time relative to the untreated cultures. Furthermore, during early exposure times cell viability decreased as BPO concentration increased in vanishing formulations; the relationship did not persist as the exposure time was increased to 27 h. The addition of tint ingredients to the formula also altered the BPO concentration-exposure time relationship.

Table 3 shows the effective time where 75% of the keratinocytes remain viable in the skin model (ET_{75}) after exposure to each of the formulations tested. ET_{75} was arbitrarily chosen from the time

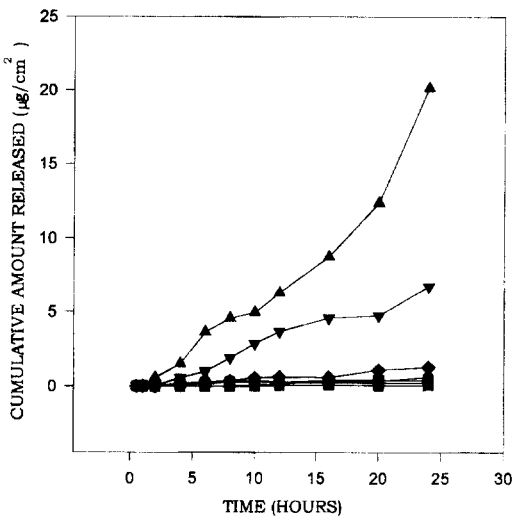

Fig. 5. Permeation of SF using tissue exposed to the receptor medium or to formulation G. Tissue exposed to the receptor medium for 6 h (■), 12 h (●), and 24 h (◆). Tissue exposed to formulation G for 6 h (▼) and 12 h (▲). Tissue exposed to saline for 12 h (□). Control (○).

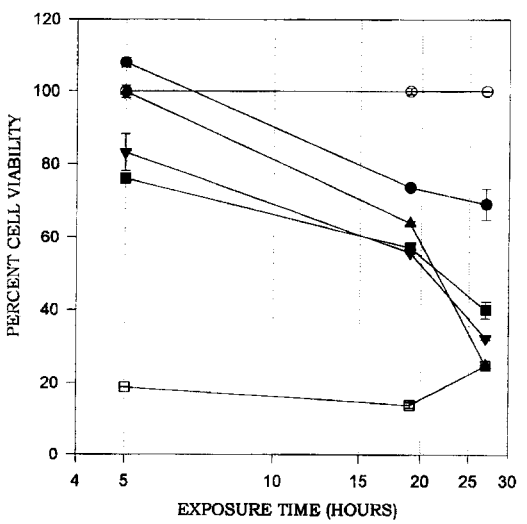

Fig. 6. Effect of vanishing formulations on cell viability over time. Untreated (○); Formulation A (●); Formulation C (▲); Formulation E (▼); Formulation G (■); 1% sodium lauryl sulfate (□).

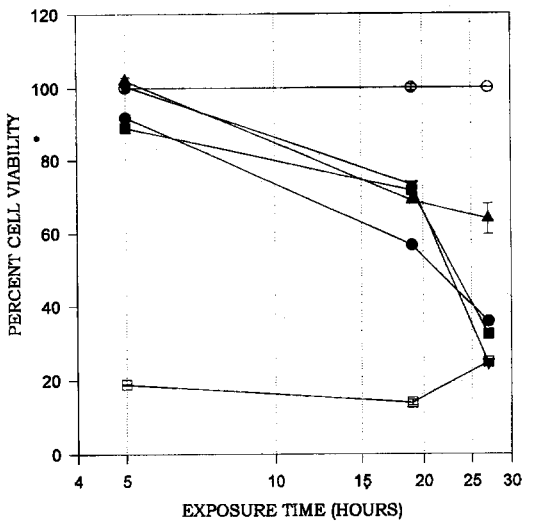

Fig. 7. Effect of tinted formulations on cell viability over time. Untreated (○); Formulation B (▲); Formulation D (▼); Formulation F (■); Formulation H (●); 1% sodium lauryl sulfate (□).

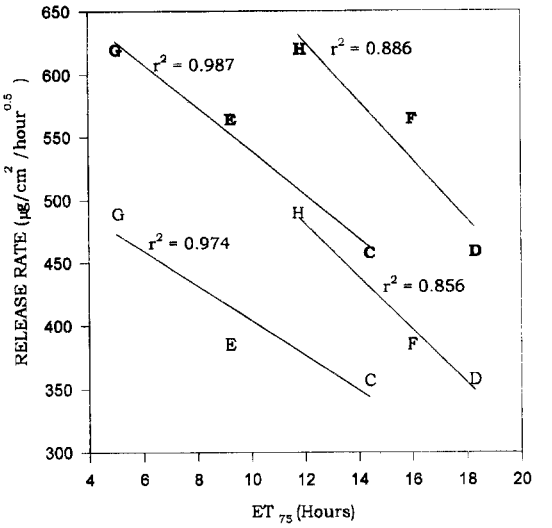

Fig. 8. Relationship between ET_{75} and release rates of various formulations. Letters refer to formulations C–H. Boldface letters: Rates obtained from silicone sheeting. Non-boldface letters: Rates obtained from tissue.

response curve of BPO-vanishing formulations where increasing BPO concentration decreased cell viability. The ET_{75} decreased as the concentration of BPO increased in vanishing formulations (Formula A > Formula C > Formula E > Formula G). Tinted formulations also exhibited decreasing ET_{75} as the concentration of BPO increased (Formula B > Formula D > Formula F > Formula H). Comparisons of ET_{75} of vanishing and tinted formulations containing the same amounts of BPO indicated that a longer exposure time was needed for tinted formulations to acquire the same cell response as vanishing formulations. The end point of this experiment, measurement of cell viability, was general and, therefore, these results could suggest that tinted formulations have an impaired ability to deliver BPO to the skin model compared to vanishing formulations. Alternatively, tinted formulations contain ingredients that could offer some protection from BPO-induced cell death without altering the tinted formulation to deliver BPO to the tissue.

Table 3
Effective time for 75% of the cells remaining viable after treatment with formulations

Formulations	ET_{75} (h)
A	18.5
B	16.5
C	14.4
D	18.3
E	9.2
F	16.0
G	5.0
H	11.8

In an attempt to compare the permeation data to the irritation results, a plot of release rates versus ET_{75} was constructed as shown in Fig. 8. As presented in the graph, cell viability was linearly related to the rate of release of BPO from the formulations. At high release rates, the time needed to reach ET_{75} was smaller. This indicated that the amount of BPO delivered from the vehicle was the primary cause of irritation. Formulations containing higher levels of BPO had higher release rates and, thus, were more irritating. This finding is in agreement with previously published results (Fulton and Bradley, 1974). Slopes computed from the regression lines obtained from the silicone sheeting and tissue data were similar. This

similarity indicated that the two barriers were comparable and that both showed a similar trend in correlating irritation to permeation. Consequently, as indicated by the data, while studying the permeation of BPO one can obtain some insight regarding the irritation of the formulation and vice versa. This could be very useful in comparing formulations with special release characteristics such as controlled-release formulations.

4. Conclusions

The similarity of the release profiles, permeation rates and diffusion coefficients suggested that the two models were comparable for measuring permeation of BPO. The use of cultured skin instead of silicone sheeting for routine lab screening might not be very advantageous due to the high cost and handling problems associated with these newly-introduced models. However, the histological similarity between cultured and human epidermis offers a great advantage over silicone sheeting, especially if the permeation of more than one active is to be determined. In this study, it was found that the use of acetonitrile/water as a receptor media did not change the permeability of the skin to a great extent. The concept of using small amounts of organic solvents in the receptor media is extremely useful and could be used with other actives with low aqueous solubility as long as the permeability of the skin does not change to a great extent. From the data presented, one can notice that the test used for irritation differentiated between formulations having different concentrations of BPO and also distinguished between vanishing and tinted formulations. It was also shown that irritation was related to the rate of permeation which indicated that BPO was the main irritant in the formula.

In conclusion, this article presented a new approach for in vitro measurement of permeation and irritation of formulations containing BPO. The methods presented are quick and simple and have a great potential to be used on a routine basis to screen formulations.

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