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Benzophenone-3: rapid prediction and evaluation using non-invasive methods of in vivo human penetration

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Abstract

The study described in this paper constitutes a practical assay system to evaluate in vivo drug penetration using two complementary non-invasive methods. An electrical capacitance test was first applied to the skin on the forearm to evaluate the hydration of the skin, and check the integrity of the stratum corneum. In the first step, the percentage absorption was measured using an occlusive and difference method; following benzophenone-3 application any residual formulation was washed off and the amount removed analyzed. In the second step, the tape stripping method—a useful procedure for selectively removing the skin's outermost layer, the stratum corneum, and measuring the stratum corneum adsorption—was performed. Under these conditions the human skin permeation of this UV-filter over four hours was near to 35% of the applied dose with the occlusive method. The amount of topically applied benzophenone-3 found in the stratum corneum after 30 min exposure using the stripping procedure was evaluated at 4% to the applied dose.

Keywords: Tape stripping method; In vivo absorption; Benzophenone-3

1. Introduction

UV-filters are commonly applied to the surface of human skin. Percutaneous absorption can be measured by a variety of methods, but despite the importance of human dermal exposure to UV-filters and pharmacological compounds, the majority of the studies are performed in-vitro with animal or synthetic membranes and 'in vivo' with animal models. Nevertheless, animal skin is often more permeable than human skin. Several analysis have shown means to predict percutaneous penetration of drug and toxic compounds solely on the basis of their physicochemical properties. Even though human studies are the 'gold standard', data available in human are limited.

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The work described here extends the scope of previous studies to include rapid in vivo studies. It is important to conduct non-invasive human studies to estimate the levels of chemical following dermal exposure. The term 'non-invasive' means 'a procedure or instrument causing minimal and only temporary changes to structure or function, and in particular, not involving pain, incision or loss of blood' [1].

The optimal characteristic for percutaneous absorption is that the permeant is reasonably soluble in both hydrophilic and hydrophobic media, expressed as the log octanol/water partition coefficient ($\log P$). This supposition is supported by data presented by Yano et al. [2], who found that the human skin permeation of salicylate and anti-inflammatory drugs over 4 h required an optimal $\log P$. The relationship between the in vivo uptake of these two series of drugs and their $\log P$, was a parabolic equation. The present study was undertaken to evaluate the skin permeability of the benzophenone-3 with the same occlusive methodology and to compare the results obtained with data found for salicylate and anti-inflammatory drugs. A tape-stripping methodology was also used to estimate the stratum corneum adsorption of benzophenone-3, at a fixed time-point. These two procedures should provide useful and complementary information for predicting the absorption and adsorption of benzophenone-3 in vivo human studies.

2. Experimental

2.1. Reagents

Benzophenone-3 (2-hydroxy-4-methoxybenzophenone), was used without further purification (stated purity 99.5%, Sigma Chemical, St. Louis, USA). Methyl alcohol (99.9%, Carlo Erba RS HPLC, Farmaitalia, Milano, Italy), for HPLC. Ethyl alcohol (absolute) and acetone (99.7%, Carlo Erba RPE-ACS, Farmaitalia, Milan, Italy), hexane and ethylene glycol (Prolabo, Fontenay s/bois France) and surfactant: octoxynol-9 (Triton X100, Sigma Chemical, St. Louis, USA). Water was freshly redistilled.

2.2. Apparatus

Analysis of benzophenone-3 was performed on a UVIKON 922 spectrophotometer (Kontron Instrument) or on a Beckman 344 LC system with a variable wavelength detector set at 287 nm. The chromatograph was equipped with a Merck Lichrosph 60RP select B (250×4.6 mm, 5 μm) column. The mobile phase was methanol–water (92:8, v/v) with a flow rate of 0.75 ml min^{-1}, the injection volume was 20 μl.

2.3. Analysis of benzophenone-3

2.3.1. Liquid chromatography

The liquid chromatographic method (LC) validated [3] was used for the determination of benzophenone-3 in stripping samples.

2.3.2. UV-spectrometry

The absorption spectrum was recorded between 200 and 330 nm. The spectrum of benzophenone-3 showed maximum at 242 and 287 nm and minimum at 230, 263 and 309 nm. The ultraviolet spectrum for benzophenone-3 was realized in hexane/ethanol mixture (33.2/66.8, v/v). The best-fit equation (at 287 nm) was $y = 0.0618x - 0.05$, where y is the optical density (OD) and x is mg l^{-1} of benzophenone-3. The R-squared statistic (R^2) indicates that the model explain 99.9% of the variation in OD.

2.4. Volunteers

After informed consent, six healthy Caucasians volunteers aged 37.3 ± 7.7 years participated in the study. The volunteers were free of dermatological disorders.

2.5. In vivo experiments: corneometer method

The measurement of skin moisture was based on a capacitance method, using a corneometer 820 (COURAGE + KHAZAKA electronic GmbH, Köln, Germany). All subjects were kept quiet for 15 min at 25 °C before capacitance measurements to prevent skin transpiration. Then measurements were performed ($n = 6$) in the in-

tern forearm, the short measuring time (1 s) prevents occlusion effects which could influence the result. Between each essay the cell was cleaned with a dry cotton swab to eliminate all residues and to obtain zero on the apparatus.

2.6. In vivo experiments: 'difference' method

The anatomical site chosen was the inner forearm near the elbow. According to the method of T. Yano et al. [2] and the recommendations of the ECVAM workshop [4], a 1.4 × 1.4 cm square was marked by hexane on the skin. To this area, 10 μl of acetone solution containing 0.5 mg (2190 nmol) of benzophenone-3 was added drop wise using a micro-syringe. Gentle blowing evaporated the solvent, and the area was covered immediately with aluminum foil. The edges were fixed with '3M' invisible adhesive tape for sealing.

The tape was removed together with the foil immediately after application (time = 0) and exactly four hours later (time = 4 h). Remaining benzophenone-3 adhering to the surface of the skin was recovered by washing with 16.6 ml of hexane and collected with the help of funnel (the aluminum foil was placed in the funnel and rinsed at same time). Hexane solution was diluted with ethanol to make exactly 50 ml. Benzophenone-3 was determined quantitatively by ultraviolet spectrometry at 287 nm. To have reliable result, this procedure was also repeated with the vehicle without benzopheneone-3 (one subject), and a blank was determined.

2.7. In vivo experiments: tape stripping method

The anatomical site chosen was the intern forearm. Volunteers (six per group) were Caucasians and free of dermatological disorders. 1000 nmoles of benzophenone-3 in 20 μl of ethylene glycol: triton X100 (90:10 v/v), were applied to the surface of the skin and spread uniformly over the whole area. An open circular cell fixed with adhesive tape delimited this area (1 cm^2). Application time was 30 min. After this time the excess substance on the applied area was quickly removed, by light cleaning with a cotton swab and rinsing twice with 500 μl ethanol followed by distilled water (500 μl) and finally light drying with a cotton swab. Next, the stratum corneum of the treated area was removed by seven successive tape strippings using '3M' invisible adhesive tape. Strippings were applied under controlled conditions over 10 s. The first sample was discarded and the following six strippings were pooled in a 50 ml vessel with 5 ml of methanol and then stirred with a magnetic bar for 30 min. The amounts of benzophenone-3 were determined by liquid chromatography. To have reliable result this procedure was also repeated (one subject) with the vehicle without benzophenone-3, and a blank was determined.

3. Results and discussion

3.1. Moisture content of the stratum corneum

The principal diffusion barrier has been well identified as stratum corneum, the integrity of which must be controlled prior to study. The hydration of the stratum corneum (the nonviable epidermis) is known to influence drug penetration of the skin and can influence the quantity of stratum corneum that is removed by a piece of tape [5]. Therefore, it was necessary to perform biophysical measurements of the SC on the area studied. Techniques for the assessment of skin hydration are often based on the electrical properties of the stratum corneum. A commonly used instrument for measurements of skin moisture is the corneometer, which detects changes in the dielectric constant of the material in contact with the probe.

In the present study, the water content of the horny layer of each volunteer before application of benzophenone-3 was estimated by capacitance measurement, and the corneometer readings (arbitrary units) are reported in Fig. 1. With a general mean of 68.2, these findings indicate an adequate degree of skin hydration and it can be concluded that all subjects had normal skin and that inter and intra-individual variations were minor.

3.2. Predicting in vivo stratum corneum penetration, 'difference' method.

Among the physicochemical factors influencing the percutaneous absorption of drugs, molecular weight, boiling point and lipophilicity play the most important role [6,7]. Small molecular size, a low boiling point and due to skin composition, both lipid and aqueous solubility with an optimal log P are required. Benzophenone-3 is an UV-filter which is characterized by a relatively low molecular weight (228.25) with a melting point of 63 °C and a log P of 3.58, calculated by Yerner et al. and Treffel et al. [8,9]. Overall, these physical properties suggest that benzophenone-3 could be a good candidate for percutaneous penetration.

Several in vitro or in vivo kinetic models for studying percutaneous penetration have been proposed, and the parabolic model was found to be the best [10,11]. Yano et al. [2] have determined a parabolic relationship between the in vivo uptake of a series of salicylate and non steroidal anti-inflammatory agents and their log P. This methodology was also used to analyze the incorporation of the same two series by Green et al. [12] and it seemed very important to us to determine if the parabolic equation for these drugs could be applied to benzophenone-3. These methods correspond to a residual analysis.

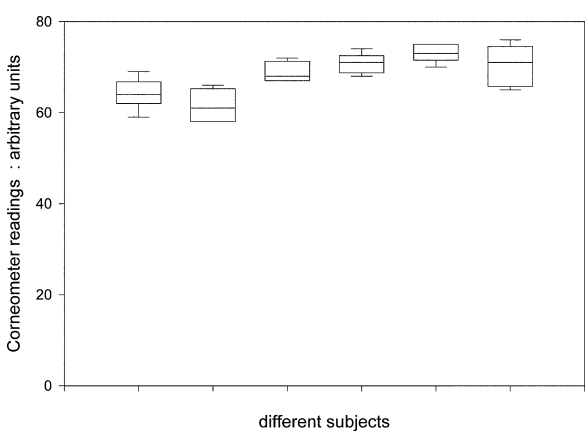

Fig. 1. The values of the skin's capacitance at the point of removal of the stratum corneum (the inner forearm); mean of results and S.D. ($n = 6$).

Table 1
Percentage absorption of benzophenone-3, the amount of drug absorbed into the skin is calculated by determining the difference between the amount recovered at time = 0 and that recoverable at 4 h, divided by the amount effectively applied (six different subjects)

	$\%Abs_{(0-4\ h)}$
Blank control (acetone without benzophenone-3)	0.60[a]
Treated subjects	47.90[b]
	30.47
	24.74[c]
	51.99
	15.49
	42.14
Mean $\%Abs_{(0\text{-}4\ h)} \pm$ S.E.M. $t_{(0.05,\ 7)}$	35.67 ± 11.60

[a] Mean of two assays 0.58 and 0.63.
[b] Mean of two assays 53.29 and 42.53.
[c] Mean of two assays 22.51 and 26.98.

The amount of drug absorbed into the skin is evaluated by determining the difference between the amount applied and immediately recoverable and that recoverable after 4 h. The %Absorption of benzophenone-3 through the skin 4 h after application was calculated from the following formula:

$$\log[Abs_{(0-4\ h)}] = \left(1 - \frac{\text{Recovery 4 h after application}}{\text{Recovery immediately after application}}\right) \times 100$$

Benzophenone-3, $\%Abs_{(0-4\ h)} = 35.67 \pm 11.60$ ($n = 8$), Table 1; which corresponds to $\log[\%Abs_{(0-4\ h)}] = 1.55$.

Without benzophenone-3 the relationship between the in vivo uptake of a series of salicylate and NSAI agents and their log P gave the following parabolic equation:

$$\log[\%Abs_{(0-4\ h)}] = -0.234(\log P)^2 + 1.144(\log P) + 0.411$$

$R^2 = 0.916$; standard error of estimate = 0.1199; Durbin Watson statistic = 2.53.

Using the data of the two series and the incorporation of benzophenone-3 data, multiple regression analysis of $\log[\%Abs_{(0-4\ h)}]$ and log P values of benzophenone-3 with the two series salicylate

and NSAIDs gave the following parabolic equation. It should be noted that this equation is very similar (Fig. 2).

$$\log[\%Abs_{(0-4\ h)}] = -0.236(\log P)^2 + 1.159(\log P) + 0.3989$$

$R^2 = 0.917$; standard error of estimate = 0.1189; Durbin Watson statistic = 2.63.

In our work, the condition of application of this UV-filter varies with each procedure. In the first method the concentration of benzophenone-3 applied was more important (twice in this case) and the exposure time was 4 h with a complete occlusion. The vehicle used (acetone) should affect the barrier properties of the skin by disruption of highly ordered structure of stratum corneum lipids. This explains the important percent of penetration. Benzophenone-3 was incorporated in two homologous series and this result indicates that partitioning from the applied vehicle to the lipophilic stratum corneum for this UV-filter was confirmed and important. These results confirm the parabolic model initially proposed by Yano et al., which implied the existence of optimal lipophilic value. The other advantage of this method is that the solvent (acetone) increased drug solubilization and an immediate evaporation occurred.

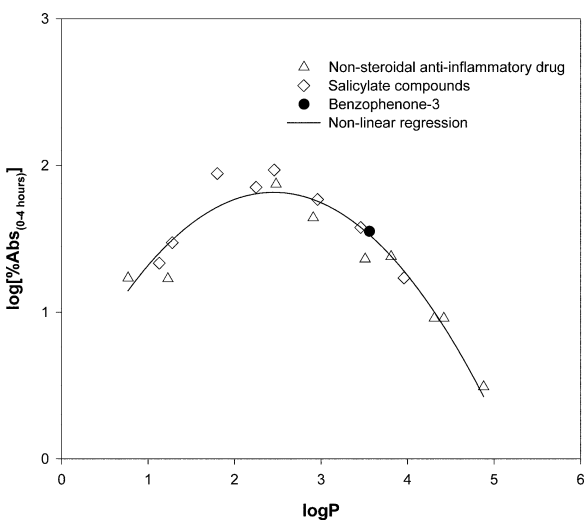

Fig. 2. The logarithm of absorption through intact skin and octanol/water partition coefficient (log P) is plotted. Incorporation of benzophenone-3 with data of a series of salicylates and non-steroidal anti-inflammatory agents studied by Yano et al.

Table 2
Stratum corneum adsorption

	Mean and S.D. of three injections (nmol cm^{-2})
Blank control (solvent without benzophenone-3)	1.784 (0.096)
Treated subjects	52.403 (0.725)
	55.669 (0.847)
	40.465 (0.256)
	39.760 (1.158)
	33.300 (0.055)
	20.199 (0.350)
Mean ± S.E.M. $t_{(0.05,\ 17)}$	40.30 ± 6.43 at 30 min
Percentage of the applied dose	4%

Amount of topically applied 1000 nmol. Benzophenone-3 found in the stratum corneum after 30 min exposure.

3.3. Evaluation of in vivo stratum corneum penetration, tape stripping method

In this method, drug penetration is estimated from the amount recovered from the stratum corneum by adhesive tape stripping following drug application. This procedure is particularly interesting for short periods of immobilization and brief skin exposure (30 min). The tape stripping method is a useful technique for selectively removing the skin's outermost layer, the stratum corneum [13,14]. The quantity of benzophenone-3 in the stratum corneum 30 min after application of this sunscreen dissolved in ethylene glycol-triton X100 was found to be 40.30 ± 6.43 nmoles cm^{-2} (Mean ± S.M.E., $t_{(0.05,\ 17)}$) (Table 2). This corresponds to the mean of the six results. This level was in agreement with those obtained in a previous study [15], where six dermatological vehicles were studied. The results of these preliminary investigations indicated that the greatest concentration of benzophenone-3 in stratum corneum was obtained with propylene glycol (58.10 ± 13.25 nmol cm^{-2}) and submicron O/W emulsion (41.08 ± 6.86 nmol cm^{-2}). We did not find any difference between capric–caprilic

triglycerides (30.60 ± 4.94 nmol cm^{-2}) and W/O emulsion (31.26 ± 4.73 nmol cm^{-2}), and the lowest levels were found with coconut oil (23.49 ± 7.07 nmol cm^{-2}) and O/W coarse emulsion (21.12 ± 3.77 nmol cm^{-2}).

It has been established that drug penetration can be estimated from the amount recovered in the stratum corneum by adhesive tape-stripping, and this procedure is often referred to as the 'reservoir technique'. Dupuis et al. [16] demonstrated a linear relationship between 'the horny layer reservoir effect' at the end of the application period (30 min) and the total penetration within 96 h, independent of the nature of the molecule tested. Several studies were first conducted on hairless rats, and then reproduced in man [17,18]. According to Rougier et al. [19] and the ECVAM workshop in their recommendations for assessing percutaneous absorption [4], knowing the chemical amount in the stratum corneum at 30 min (X), the linear correlation $Y = 1.83X - 0.52$ ($r = 0.97$, $P < 0.001$) enables calculation of the total amount likely to penetrate over a period of 96 h (Y). Under these conditions, the predicted 4 days total percutaneous absorption for benzophenone-3 was 73.22 nmol cm^{-2}.

In this procedure the compound was applied to the skin for a limited short time (30 min) without occlusion. Only the amount of chemical present in the upper layer of the immediately stripped stratum corneum was analyzed. This explains the difference of percentage absorbed between the two methods.

The first procedure is used to confirm correlation between percutaneous penetration and lipophilicity of the studied compound and the comparison with two homologous series (salicylate and non steroidal anti-inflammatory agents). The second procedure without occlusion will be predictive of the bioavailability of benzophenone-3 when the skin is undisturbed. This procedure mimics the 'in-use' conditions. The total quantity of the substance that penetrated over a 4-day period was determined from the quantity present in the stratum corneum at the end of the application using the linear correlation linking these two parameters. [14].

4. Conclusion

In this work, 'difference' and 'tape stripping' non-invasive sampling methods have been tested for measuring dermal exposure to a sunscreen (benzophenone-3). The results demonstrate the ability of this UV-filter to act within the stratum corneum, and confirm that under our operating conditions the amount of benzophenone-3 crossing the skin is near 35%. The amount of topically applied Benzophenone-3 found in the stratum corneum after 30 min exposure was evaluated at 4% of the applied dose. These two inexpensive methods are very interesting because they require small amounts of active drug, a limited area and a relatively rapid time of exposure with a non-invasive procedure on human skin. These two methods are clearly complementary in this field. The non-invasive nature of these approaches, together with the fact that the methods used were conducted in vivo on humans increases the potential utility of these results. Considering the importance of percutaneous absorption in this field, more experimental data on other UV-filters are clearly needed to investigate the undesirable feasibility of delivering sunscreens across the skin.

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