ELSEVIER

European Journal of Pharmaceutics and Biopharmaceutics 46 (1998) 279–283

European Journal of Pharmaceutics and Biopharmaceutics

Research paper

# In vitro percutaneous penetration through hairless rat skin: influence of temperature, vehicle and penetration enhancers

Peter Clarys[a,*], Katrien Alewaeters[a], Ann Jadoul[b], Andre Barel[a], Rui Oliveira Manadas[b], Véronique Préat[b]

[a]*General and Biological Chemistry, Faculty of Physical Education and Physiotherapy, Vrije Universiteit Brussel, Brussels, Belgium*
[b]*Unité de Pharmacie Galénique, Université Catholique de Louvain, Brussels, Belgium*

Received 9 February 1998; accepted 6 April 1998

**Abstract**

Theoretically, a positive relation is expected between skin temperature and the percutaneous penetration of topically applied substances. The aim of the present study was to evaluate the influence of the temperature on the in vitro percutaneous penetration of dihydrotestosterone. Hairless rat skin was mounted in static diffusion cells placed in a water bath at different temperatures (28.6, 35.1 and 38.2°C, respectively). Different vehicles were tested as well as the addition of penetration promoting molecules such as oleic acid and limonene. A saline buffer was used as the receptor phase. Penetration through the skin was evaluated by means of scintillation counting of the radiolabelled dihydrotestosterone. Experiments were followed for a period of 29 h. The total amount of penetrant, dihydrotestosterone, as well as the flux, were calculated from these kinetics. Our results demonstrate a temperature effect with the highest penetration at 38.2°C. The vehicle was also found to influence the penetration of dihydrotestosterone in a significant way. Furthermore, limonene presented better penetration promoting properties compared with oleic acid. 

*Keywords:* Temperature; Vehicle; Enhancers; Percutaneous penetration; Dihydrotestosterone

## 1. Introduction

The diffusion constant for diffusion processes across a homogenous membrane increases with increasing temperature [1]. As a consequence, a positive relation is expected between skin temperature and the percutaneous penetration of topically applied substances. Hence, the penetration of active substances from transdermal drug delivery devices may be affected in a significant way by variations of skin temperature. These variations can be caused, for instance, by the occlusive nature of these patches [2] or by physical exercise by the patients while wearing the patches [3,4]. Reports on contact irritation processes point to the importance of the temperature in the provocation of cutaneous irritation, mainly explained by an increased penetration of the applied irritants [5,6].

In vivo penetration studies, carried out on human volunteers demonstrated the influence of temperature on percutaneous absorption of substances under physiological conditions [1,3]. However, since these in vivo experiments measure a total process they are unable to distinguish between effects due to the weakened epidermal barrier function and effects due to the increased perfusion of the microcirculation. As argued by Gay et al. [7] lipid transitions in the human stratum corneum occurring near normal skin temperature may affect the permeability of the barrier properties in a significant way. On the other hand, Auclair et al. [8], demonstrated the importance of the local blood flow in the percutaneous absorption process.

* Corresponding author. Vrije Universiteit Brussel, Faculty for Physical Education and Physiotherapy, Department of General and Biological Chemistry, Room 6G304, Pleinlaan 2, 1050 Brussel, Belgium. Tel.: +32 2 6293551; fax: +32 3 6292276; e-mail: pclarys@vub.ac.be

0939-6411/98/$19.00 
*PII* S0939-6411(98)00044-7

The aim of the present study was to investigate the influence of temperature variations, within a physiological range, on the percutaneous penetration process. Using an in vitro diffusion system with freshly excised hairless rat skin, the effect of temperature variations on the diffusional properties of the barrier lipids was assessed without interference of the blood circulation [7,9]. Application of the penetrant in different vehicles with and without penetration enhancers enabled us to estimate combined effects of temperature and vehicle, temperature and penetration enhancers as well as vehicle and penetration enhancer. Two penetration enhancers with a different mode of action were used in our experiments [10–12].

## 2. Materials and methods

### 2.1. Chemicals

5$\beta$-dihydrotestosterone and the corresponding tritiated compound, 5$\alpha$-dihydro(1,2,4,5,6,7)[$^3$H]testosterone (100 $\mu$Ci/ml), were purchased from Sigma (St Louis, MO, USA) and Amersham (Life Science, Ghent, Belgium), respectively.

Ethanol (96% extra pure) was purchased from Merck (Merck Belgolabo, Overijse, Belgium) and propylene glycol from UCB (Leuven, Belgium). Oleic acid and D-limonene were ordered from Sigma. Transcutol was a gift from Mosselman n.v./s.a. (Belgium). All solutions were prepared in ultra pure water (Sation 9000, Barcelona, Spain).

### 2.2. Apparatus and procedures

The diffusion experiments were performed in in vitro vertical diffusion chambers made of two compartments. The donor and receptor compartments were separated by freshly excised hairless rat skin (full thickness abdominal skin; mutant Iops hairless rat, Iffa Credo, St Germain les Arbresles, France), with the epidermis facing the donor compartment. For all experiments, the lower compartment (receptor) was filled with 2.5 ml PBS (w/o calcium and magnesium, GibcoBRL Life Technologies). The donor chamber was filled with 0.4 ml 5$\beta$-dihydrotestosterone solution. 5ß-dihydrotestosterone (10 mg/ml) was introduced in: ethanol/water 50/50 v/v; propylene glycol; oleic acid (10% v/v in propylene glycol); limonene (2% v/v in propylene glycol); transcutol; oleic acid (10% v/v in transcutol).

Samples (0.2 ml) were taken at regular intervals from the receptor phase up to 29 h after starting the procedure. Each sample was placed in a separate scintillation vial and mixed with liquid scintillation cocktail (Ready Safe™, Liquid Scintillation Cocktail, Beckman Instruments, USA). The radioactivity was measured by $\beta$-counting (Wallac 1410 LKB Pharmacia).

Experiments were carried out at three physiological temperatures with the cells placed in water baths of 28.6 ± 0.5°C, 35.1 ± 0.4°C and 38.2 ± 0.5°C, respectively. Temperature on the skin surface (donor compartment) was measured using a contact thermometer (Testoterm 9010®; Testoterm, Lenzkirch, Germany).

The experimental protocol was approved by the Ethical Committees of the two involved universities.

### 2.3. Data treatment and statistics

The cumulative amount of dihydrotestosterone in the receptor compartment was plotted as a function of time. The steady state fluxes of dihydrotestosterone ($\mu$g/cm$^2$ per h) were determined from the slope of the linear part of the plot using linear regression analysis ($R > 0.95$).

The amount of penetrant in the receptor compartment at the end of the experiment under the different (1) temperature; (2) vehicle; (3) enhancer conditions was compared using the ANOVA procedure. This procedure was only carried out after testing for normality (Kolmogorov Goodness of Fit test). The flux in every cell was compared as a function of the temperature, vehicle and enhancer using the ANOVA procedure. The significance level was set at 5%.

## 3. Results

### 3.1. Influence of temperature

A graphical representation of the cumulative amount of 5$\beta$-dihydrotestosterone found in the receptor phase after diffusion from the propylene glycol vehicle at the different experimental temperatures is given in Fig. 1. These kinetics indicate a marked temperature effect. Increasing the temperature resulted in increased penetration of dihydrotestosterone. Similar profiles were detected for the other vehicles, ethanol/water and transcutol, respectively. When comparing the amount of penetrant in the receptor fluid at the end of the experiment, we found a significant temperature effect for

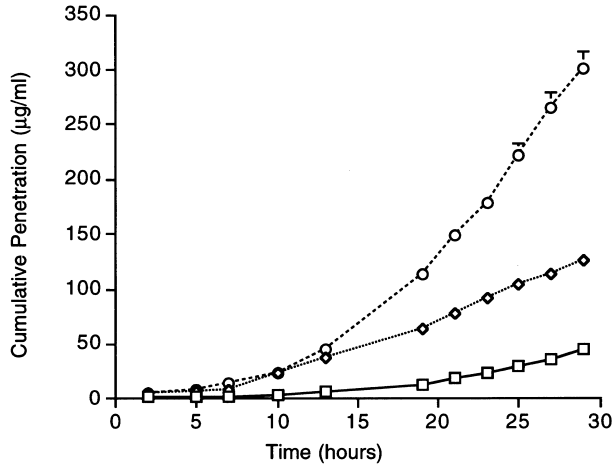


Fig. 1. Cumulative amount of 5$\beta$-dihydrotestosterone ($\mu$g/ml) in the receptor fluid after penetration from the propylene glycol vehicle as measured at 28.6°C (□); 35.1°C (◇) and 38.2°C (○). Mean ± SE.

Table 1
Cumulative amount of 5$\beta$-dihydrotestosterone ($\mu$g/ml) found in the receptor fluid after 29 h (mean ± SE)

| | Temperature (°C) | | | |
|---|---|---|---|---|
| Vehicle | 28.6 | 35.1 | 38.2 | ANOVA[a] |
| Ethanol/water (*n*) | 410 ± 70 (9) | 493 ± 41 (5) | 563 ± 80 (10) | $F = 1.20$, $P = 0.321$ |
| Propylene glycol (*n*) | 45 ± 12 (11) | 126 ± 48 (10) | 301 ± 71 (13) | $F = 6.36$, $P = 0.005$ |
| Transcutol (*n*) | 25 ± 16 (5) | 14 ± 2 (6) | 68 ± 28 (8) | $F = 1.84$, $P = 0.190$ |
| ANOVA[b] | $F = 23.28$, $P < 0.001$ | $F = 25.33$, $P < 0.001$ | $F = 10.99$, $P < 0.001$ | |

*n*, number of cells run under that specific condition.
[a]ANOVA results of the temperature effect.
[b]ANOVA results of the vehicle effect.

the propylene glycol vehicle. The two other vehicles (ethanol/water and trancutol) showed a similar rank order for the amount of penetrated substance as a function of the temperature. However, the differences were not statistically significant.

### 3.2. Influence of the vehicles

When comparing the different vehicles, it was found that the ethanol/water donor phase provoked the highest penetration at all temperatures (28.6, 35.1 and 38.2°C, respectively). The propylene glycol vehicle provoked lower penetration profiles, while a very weak penetration was detected for the transcutol vehicle. Statistically we found a significant vehicle effect at all temperatures (Table 1).

### 3.3. Influence of the penetration enhancers

The combination of propylene glycol with the penetration enhancers oleic acid and limonene resulted in higher penetration profiles compared to application in the vehicle alone. This was found for all tested temperatures except for the propylene glycol vehicle with oleic acid at 38.2°C (Table 2). Regarding the effect of the two enhancers, higher penetration values were recorded for limonene compared to oleic acid, especially at 35.1°C and 38.2°C (with $P = 0.002$ and $P = 0.038$, respectively).

For the transcutol vehicle we only used oleic acid as a penetration enhancer. Under these conditions we found a significantly increased penetration for 5$\beta$-dihydrotestosterone at 35.1 and 38.2°C while no effect of oleic acid was observed at 28.6°C (Table 3).

### 3.4. Flux

The dihydrotestosterone fluxes ($\mu$g/cm$^2$ per h) as calculated for the different experimental conditions are represented in Fig. 2. Although less pronounced, these data are in complete agreement with the data referring to the amount of dihydrotestosterone measured in the receptor fluid at the end of the experiment.

## 4. Discussion

Our results indicate a significant temperature effect on the penetration of 5ß-dihydrotestosterone through freshly excised full thickness rat skin. The amount of 5$\beta$-dihydrotestosterone in the receptor fluid increased as a function of the temperature. Besides a significant temperature effect, a significant vehicle effect was detected. Highest penetration values were found for the ethanol/water vehicle, lower values for the propylene glycol vehicle and the lowest for the transcutol vehicle. Moreover, when comparing the penetration enhancing molecules it was found that limonene was much more effective than oleic acid.

Our findings corroborate the literature concerning in vitro experiments on animal and human skin [5,13]. Indeed, an in vitro temperature effect on the penetration of sodium lauryl sulfate was reported by Emilson et al. [5]. However, these

Table 2
Cumulative amount of 5$\beta$-dihydrotestosterone ($\mu$g/ml) found in the receptor fluid after 29 h (mean ± SE)

| | Temperature (°C) | | | |
|---|---|---|---|---|
| Propylene glycol | 28.6 | 35.1 | 38.2 | ANOVA[a] |
| No enhancer (*n*) | 45 ± 12 (11) | 126 ± 48 (10) | 301 ± 71 (13) | $F = 6.36$, $P = 0.005$ |
| Oleic acid (*n*) | 221 ± 53 (10) | 240 ± 43 (10) | 340 ± 57 (9) | $F = 1.53$, $P = 0.236$ |
| Limonene (*n*) | 288 ± 78 (13) | 539 ± 69 (12) | 597 ± 87 (13) | $F = 4.39$, $P = 0.020$ |
| ANOVA[b] | $F = 4.64$, $P = 0.017$ | $F = 14.53$, $P < 0.001$ | $F = 4.75$, $P = 0.016$ | |

*n*, number of cells run under that specific condition.
[a]ANOVA results of the temperature effect.
[b]ANOVA results of the vehicle effect.

Table 3
Cumulative amount of 5β-dihydrotestosterone (μg/ml) found in the receptor fluid after 29 h (mean ± SE)

| | Temperature (°C) | | | |
|---|---|---|---|---|
| Transcutol | 28.6 | 35.1 | 38.2 | ANOVA[a] |
| No enhancer (*n*) | 25 ± 16 (5) | 14 ± 2 (6) | 68 ± 28 (8) | $F = 1.84$, $P = 0.190$ |
| Oleic acid (*n*) | 15 ± 3 (5) | 40 ± 9 (8) | 294 ± 96 (8) | $F = 6.85$, $P = 0.006$ |
| ANOVA[b] | $F = 0.365$, $P = 0.562$ | $F = 5.74$, $P = 0.034$ | $F = 5.79$, $P = 0.030$ | |

*n*, number of cells run under that specific condition.
[a]ANOVA results of the temperature effect.
[b]ANOVA results of the vehicle effect.

authors used a much broader range of temperatures ranging from 22 to 40 and 60°C, respectively. These extreme temperatures are physiologically unacceptable and hence inapplicable under in vivo conditions [14]. As found in our experiment using rat skin instead of excised human skin, the temperature effect is already visible in a much smaller range of temperature variations. These variations are physiologically acceptable and can be used for in vivo experimentation on human volunteers [1,3]. In the reports on these in vivo experiments [1,3,4] it was hypothesized that the increased penetration was due to a combined effect on the barrier function and the increased perfusion of the skin microcirculation. Our results, using a model without a perfused microcirculation, demonstrated a substantial contribution of the barrier function, with a weaker barrier function at higher temperatures. This finding is completely in agreement with the findings of Gay et al. [7]. In their experiments, stratum corneum lipid transitions were found to occur from 35°C on. Alteration of the lipid phase is believed to facilitate diffusional processes with a facilitated diffusion through a more liquid medium [7]. The results obtained in our experiment demonstrated significant alterations in the barrier function due to the changes in the experimental temperature. It therefore seems reasonable to assume that these alterations were equally induced by structural changes of the diffusion medium.

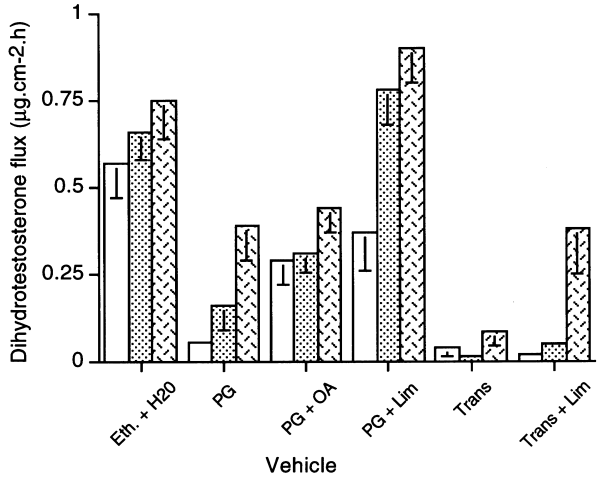


Fig. 2. Dihydrotestosterone flux (μg/cm$^2$ per h) as calculated for the different experimental conditions. (□) at 28.6°C; (dotted box) at 35.1°C (stipled box) at 38.2°C. Mean ± SE.

The influence of the vehicle on the percutaneous penetration process as found in our experiment has been extensively reported in the literature (for a review see Rougier et al. [15]).

The observed increased penetration when using the penetration enhancing molecules, oleic acid or limonene, is also in accordance with the literature [10–12]. The penetration enhancing effect of limonene was stronger than the effect of oleic acid. According to the same authors, this can be explained by their different mode of action. Oleic acid acts only at the level of the intercellular lipids. On the other hand, limonene is not only active at that level but it improves the partitioning of substances in the stratum corneum as well [16,17].

Our results point equally to a combined effect of temperature and penetration enhancers. Indeed, a cumulative effect was found when using oleic acid and limonene, respectively, in combination with increased temperature. This finding again advocates the importance of the diffusional processes. The combination of increased fluidity by the temperature effect and disturbance of the ordered lipid bilayers by the insertion of penetration enhancing molecules is shown to decrease the diffusional resistance in a significant way [7].

In conclusion, our results indicate the marked influence of temperature on the in vitro penetration of dihydrotestosterone through rat skin. For the vehicles where a low penetration rate was detected, we found a synergistic effect of increased temperature and the use of penetration enhancing molecules, resulting in a significant increase in dihydrotestosterone penetration.

Our in vitro findings, together with the results of others [1–6] indicate that even small skin temperature variations can have a significant influence on the penetration of topically applied substances. These variations should be taken into account in transdermal therapy for the following reasons: occlusion of the skin causes a significant increase in skin temperature [2]; marked skin temperature variations exist in function of the body region [18]; and, finally, because significant temperature changes can be induced by environmental conditions or physiological exercise [4].

## References


[1] P. Clarys, B. Gabard, A.O. Barel, The effect of the temperature on the in vivo percutaneous penetration process, in: K.R. Brain, V.J. James, K.A. Walters (Eds.), Prediction of Percutaneous Penetration: Methods, Measurements, Modelling, STS Publishing, Cardiff, UK, 1996, pp. 278–281.

[2] D.A. Bucks, H.I. Maibach, R.H. Guy, Occlusion does not uniformly enhance penetration in vivo, in: R.L., Bronaugh, H.I. Maibach, (Eds.), Percutaneous Absorption. Mechanisms-Methodology-Drug Delivery, Marcel Dekker, New York, 1989, pp. 77–93.

[3] T.O. Klemsdal, K. Gjesdal, J.E. Bredesen, Heating and cooling of the nitroglycerin patch application area modify the plasma level of nitroglycerin, Eur. J. Clin. Pharmacol. 43 (1992) 625–628.

[4] R. A Lefebvre, M.G. Bogaert, O. Teirlynck, A. Sioufi, J.P. Dubois, Influence of exercise on nitroglycerin plasma concentrations after dermal application, Br. J. Clin. Pharmacol. 30 (1990) 292–296.

[5] A. Emilson, M. Lindberg, B. Forslind, The temperature effect on in vitro penetration of sodium lauryl sulfate and nickel chloride through human skin, Acta Derm. Venereol. (Stockh.) 73 (1993) 203–207.

[6] E. Berardesca, G.P. Vignoli, F. Distante, P. Brizzi, G. Rabbiosi, Effects of water temperature on surfactant-induced skin irritation, Contact Dermatitis 32 (1995) 83–87.

[7] C.L. Gay, R.H. Guy, G.M. Golden, V.H.W. Mak, M.L. Francoeur, Characterization of low-temperature (i.e. $<65°C$) lipid transitions in human stratum corneum, J. Invest. Dermatol. 103 (1994) 233–239.

[8] F. Auclair, M. Besnard, C. Dupont, J. Wepierre, Importance of blood flow to the local distribution of drugs after percutaneous absorption in the bipediculated dorsal flap of the hairless rat, Skin Pharmacol. 4 (1991) 1–8.

[9] V. Préat, S. Thysman, Transdermal iontophoretic delivery of sufentanil, Int. J. Pharm. 96 (1993) 189–196.

[10] B.-W. Barry, Penetration enhancers; mode of action in human skin, Pharmacol. Skin 1 (1987) 121–137.

[11] B.-W. Barry, Mode of action of penetration enhancers in human skin, J. Controlled Release 6 (1987) 85–97.

[12] J. Hadgraft, W.A. Walters, Skin penetration enhancement, J. Dermatol. Treat. 5 (1994) 43–47.

[13] W.G. Reifenrath, T.S. Spencer, Evaporation and penetration from skin, in: R.L. Bronaugh, H.I. Maibach (Eds.), Percutaneous Absorption. Mechanisms-Methodology-Drug Delivery, Marcel Dekker, New York, 1989, pp. 313–334.

[14] C.R. Behl, S. Kumar, W. Malick, S. Del Terzo, W.I. Higuchi, V. Srinivasan, Iontophoretic drug delivery: effects of physicochemical factors on the skin uptake of drugs, in: R.L. Bronaugh, H.I. Maibach, (Eds.), Percutaneous Absorption. Mechanisms-Methodology-Drug Delivery, Marcel Dekker, New York and Basel, 1989, pp. 603–631.

[15] A. Rougier, D. Dupuis, C. Lotte, H.I. Maibach, Stripping method for measuring percutaneous absorption in vivo, in: R.L. Bronaugh, H.I. Maibach, (Eds.), Percutaneous Absorption Mechanisms-Methodology-Drug Delivery, Marcel Dekker, New York and Basel, 1989, pp. 415–434.

[16] A.C. Williams, B.W. Barry, The use of terpenes as skin penetration enhancers, in: R.C. Scott, R.H. Guy, J. Hadgraft, (Eds.), Prediction of Percutaneous Penetration Methods, Measurements, Modelling (Vol. 1), IBC Technical Services, London, 1990, pp. 224–230.

[17] B.M. Magnusson, P. Runn, L.D. Koskinen, Terpene-enhanced transdermal permeation of water and ethanol in human epidermis, Acta Derm. Venereol. (Stockh.) 77 (1997) 264–267.

[18] P. Clarys, I. Manou, A.O. Barel, Relationship between anatomical skin site and response to halcinonide and methyl nicotinate studied by bioengineering techniques, Skin Res. Technol. 3 (1997) 161–168.