

Topical D-vitamins: multiparametric comparison of the irritant potential of calcipotriol, tacalcitol and calcitriol in a hairless guinea pig model

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The irritant potential of calcipotriol, $1\alpha,24$ -dihydroxyvitamin D_3 (tacalcitol) and $1\alpha,25$ -dihydroxyvitamin D_3 (calcitriol) was compared in a hairless guinea pig model. Randomized, occlusive patch testing for 2 days was used. Each group of 8 animals was tested simultaneously with the 3 substances and a placebo vehicle. 3 dose levels i.e. 500 $\mu\text{g/ml}$, 50 $\mu\text{g/ml}$ and 5 $\mu\text{g/ml}$ were used. Test sites were evaluated at day 2 (2 h after removal of the patches) and again at day 3. Evaluation was blinded and based on a multiple parameter assessment of skin irritancy, comparing clinical scoring, skin perfusion using high resolution laser Doppler image scanning, skin colour (a^* , Minolta ChromaMeter) and skin thickening (20 MHz ultrasound) indicating oedema. Skin biopsies were taken for histological preparation and assessment of epidermal hyperplasia. No difference was observed between the irritant potential of calcipotriol, tacalcitol and calcitriol based on clinical scoring as well as objective non-invasive measuring techniques. All 3 substances showed a dose-dependent and equal increase in clinical irritation score, cutaneous blood flow, skin colour and epidermal hyperplasia. The cutaneous inflammatory reaction was dominated by vasodilation and increased cutaneous perfusion. Oedema formation was only seen at the highest dosages tested. Skin barrier damage was not induced as TEWL remained unaffected. The hairless guinea pig appears a valid model to test irritancy of topical D-vitamins since the same profile of irritancy was previously established in humans for 2 of the compounds tested, calcitriol and calcipotriol.

Key words: calcipotriol; $1\alpha,24$ -dihydroxyvitamin D_3 (tacalcitol); $1\alpha,25$ -dihydroxyvitamin D_3 (calcitriol); skin irritation; in vivo; guinea pigs; non-invasive methods. © Munksgaard, 1997.

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$1\alpha,25$ -dihydroxyvitamin D_3 (calcitriol) and its 2 synthetic analogues calcipotriol and $1\alpha,24$ -dihydroxyvitamin D_3 (tacalcitol) have been introduced for topical use in psoriasis. Both calcipotriol and tacalcitol are found equipotent with calcitriol in their affinity for the vitamin D receptor and in their effects on keratinocyte growth in vitro (1–5). The dose inducing hypercalcemia in rats is similar for tacalcitol and calcitriol (5). Compared with calcitriol, calcipotriol is 100 to 200 $\times$ less potent in producing hypercalcemia and hypercalciuria in rats (1, 6), probably due to fast elimination and biotransformation into inactive metabolites after reaching the systemic circulation (7, 8). Thus, calcipotriol has an especially favourable therapeutic index. The aim of the present study was to compare the irritative potential of the three substances in a hairless guinea pig model developed in our laboratory. The guinea pigs are hairless except for

continuous hair growth at the nose and feet. One of the advantages of using a hairless species model is that injuries caused by shaving and depilation can be avoided. Assessment of skin irritation was based on a multiple parameter assessment including both clinical scoring, non-invasive measuring techniques and studies of induction of epidermal hyperplasia.

Material and Methods

Animals

24 female hr/hr guinea pigs (Charles Rivers Laboratories, Germany) weighing about 4–500 g were used in the study. The guinea pigs were placed singly in Macrolon[®] cages with free access to a commercial rodent diet (Altromin[®]) and to tap water with added ascorbic acid. The animals were acclimatized for 1 week before initiation of the

study. A 12 h light/12 h dark cycle was maintained and temperature and humidity in the animal room was recorded by a thermohygrograph.

Test solutions

Calcipotriol, $1\alpha,24$ (OH) $_2$ D $_3$, $1\alpha,25$ (OH) $_2$ D $_3$ in concentrations 5 μ g/ml, 50 μ g/ml and 500 μ g/ml were prepared in isopropanol buffered with citrate to pH $\approx$ 8.5. A vehicle solution without added drug was also included as placebo.

Patch testing procedure

The animals were divided into 3 groups each consisting of 8 animals. The guinea pigs in each test group were tested simultaneously with all 3 test substances in a defined concentration and with the solution vehicle. 2 test sites were marked on each dorsal flank of the animals. In order to remove the influence of an effect caused by location of treatment, the treatments were randomly assigned to locations in an as much as possible balanced way. For each group of 8 animals 2 different 4 $\times$ 4 latin squares were used.

60 μ l of each test preparation was applied on a filter disc fitted into a large Finn Chamber[®] (Epitest, Helsinki, Finland) and placed on the skin. Fixation of the chamber was done with Scanpor[®] tape and a final dressing consisting of tube gauze covered with Bend-A-Rete[®] (an elastic, tubular net bandage). Test chambers were removed at day 2 and readings of test sites were done 2 hours after removal of the patches and again at day 3.

Test evaluation was blinded and included clinical scoring of erythema and edema and objective measurement of cutaneous blood flow, skin colour, skin thickness and transepidermal water loss. Details about each method is given in the following text.

Clinical scoring

Clinical scoring of erythema and edema of test sites was done according to Draize et al. (9) The scoring scale is shown in Table 1. The reactions were assessed visually and palpably. A final mean score for each formulation was obtained by dividing the total score for the group by the number of animals in the group.

Laser Doppler image scanning

The blood flow in the skin was measured using a laser Doppler perfusion imager[®] (LDI system, Lisca Development, Sweden). The equipment uses a low power He-Ne laser to scan the tissue. The

Table 1. Clinical evaluation of skin reactions according to Draize et al. (9)

<i>A. Erythema and eschar formation</i>	
No erythema	0
Very slight erythema (barely perceptible)	1
Well defined erythema	2
Moderate to severe erythema	3
Severe erythema (beet redness) to slight eschar formation	4
Total possible erythema score	4
<i>B. Edema formation</i>	
No edema	0
Very slight edema (barely perceptible)	1
Slight edema (edge of area well defined by definite raising)	2
Moderate edema (area raised approximately 1 mm)	3
Severe edema (raised more than 1 mm and extending beyond area of exposure)	4
Total possible edema score	4
Total possible score for primary irritation	8

laser beam move step by step over an area of interest making a two dimensional horizontal scanning of the tissue without touch. At each measurement site the He-Ne laser beam penetrates the skin to a median depth of a few hundred micrometers. The Doppler shift of the reflected light depends on the flux of red blood cells. Precautions and more details about the equipment have been described elsewhere (10, 11).

3 scans were made within each test area using this method and the mean perfusion value $\pm$ standard deviation was subsequently measured based on a total of 224 individual measurement points. Ambient light was switched off during scanning. The parameter settings during measurements were: Image format: 32 sites $\times$ 32 sites; Resolution: high; Distance to test site: 16.5 cm.

The spatial resolution based on this set-up was 0.73 mm² being 0.94 mm in the x-axis and 0.78 mm in the y-axis.

Skin colour

Skin erythema was measured by chromametry (Minolta ChromaMeter CR-300, Osaka, Japan) with tristimulus analysis of the colour of the skin according to the CIE system. Equipment and measuring principles has previously been described (12). 3 measurements were carried out within the individual sites. The colour of the skin was measured by the parameter a* and expressed as the median of 3 observations.

Transepidermal water loss (TEWL)

An Evaporimeter EP-1[®] (Servo-Med, Kinna, Sweden) with protection cover (screen and grid)

was used. Measurements were done with precautions taken according to Pinnagoda et al (13). Measurements were performed with the animal placed in an incubator for air convection control. 3 measurements were carried out on each test site using printer registration. The mean TEWL value and standard deviation was calculated.

Skin thickness

Measurement of skin edema was done by ultrasound scanning of the skin using the Dermascan-C® (Cortex Technology, Hadsund, Denmark). The scanner is a 20 MHz high frequency scanner. The method is described in details elsewhere (14). Measurements were made using the C-probe and a standard set-up with a linear gain setting at 23. 1 scan was taken within each site. Image analysis of scans comprised measurement of skin thickness using the A-scan mode of the Dermascan-C standard software. 3 measurements were carried out

within each scan and the mean value and standard deviation was calculated.

Histology (epidermal thickness)

At day 3, immediately after sacrifice, an 8 mm punch biopsy was taken from the central location of each site. Biopsies were fixed in 10% neutral phosphate buffered formalin, embedded in paraffin wax, sectioned in 5 μ m thick slices and stained with haematoxylin and eosin (H&E). A Nikon LABOPHOT 2 microscope was used at magnification 400 $\times$. Epidermal thickness on 6 slices from each biopsy was measured and the mean thickness and standard deviation calculated.

Statistical analysis

The comparison between the treatments in each group was made using a test for paired data, i.e. repeated measures analysis of variance. Significance level was $p < 0.05$.

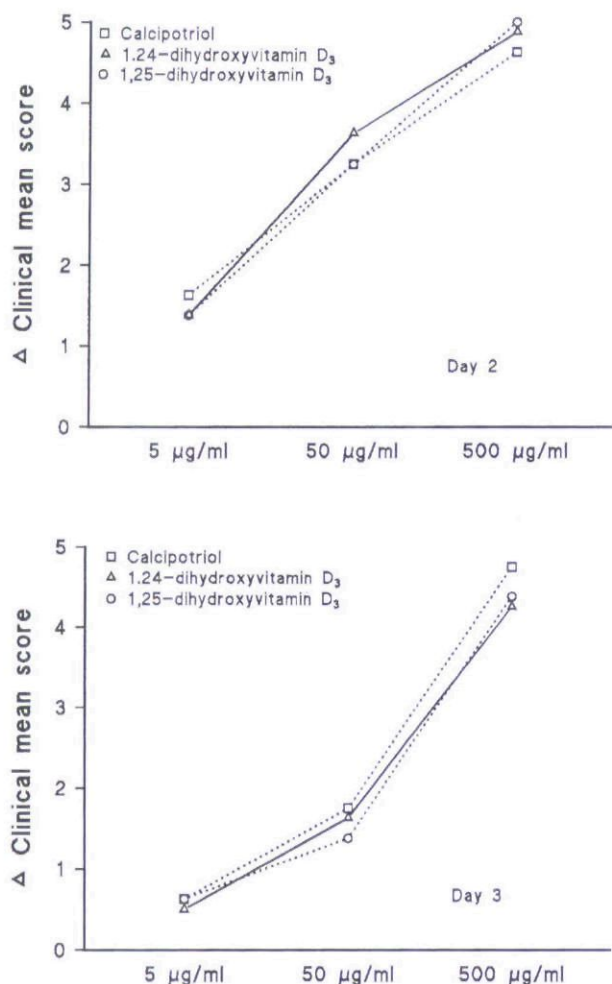


Fig. 1 Δ Clinical mean score of irritation as a function of concentrations of substances applied ($n=8$ for each concentration).

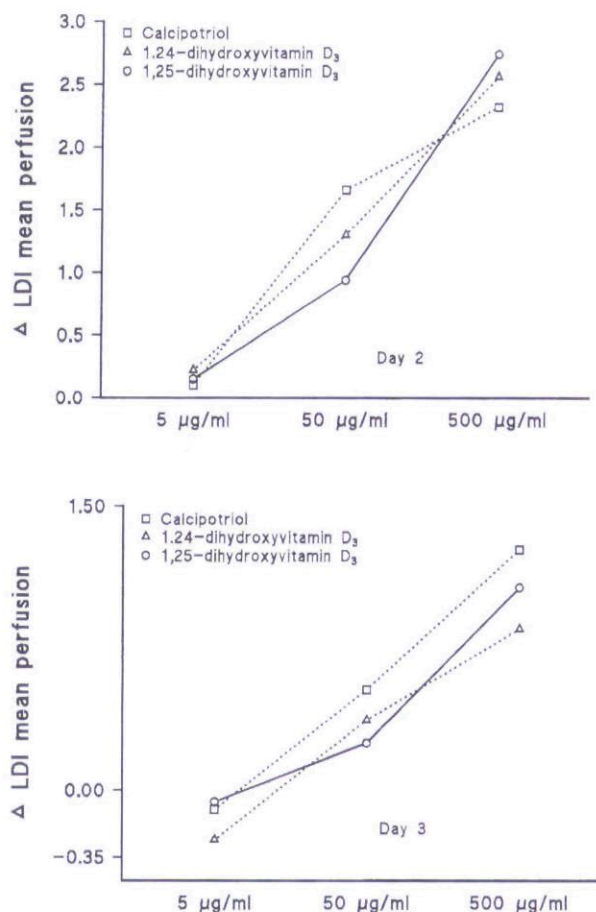


Fig. 2 Δ LDI mean blood perfusion as a function of concentrations of substances applied ($n=8$ for each concentration).

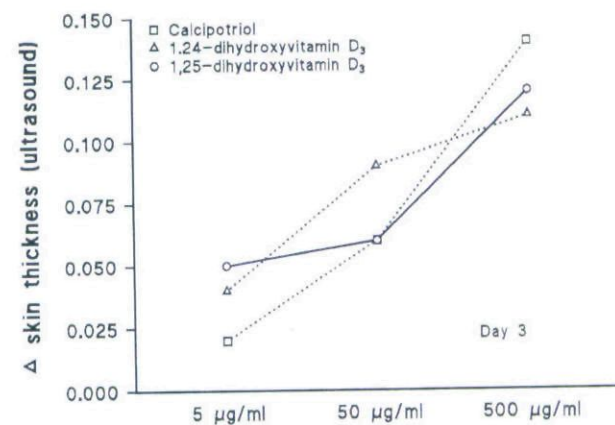
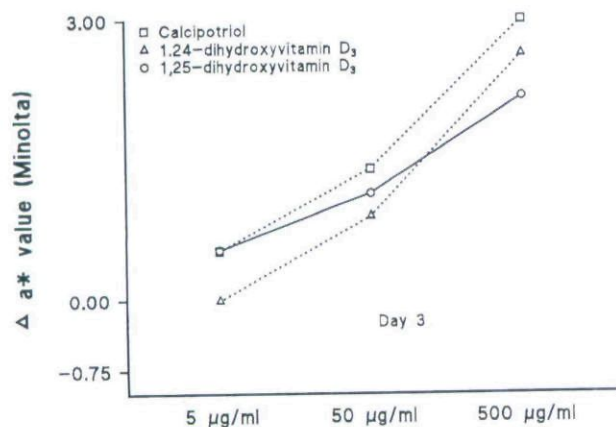
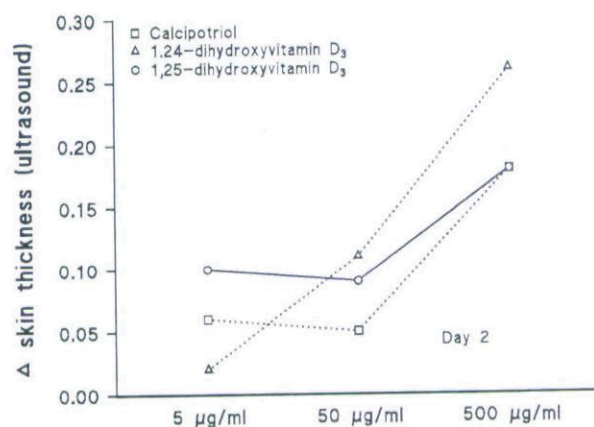
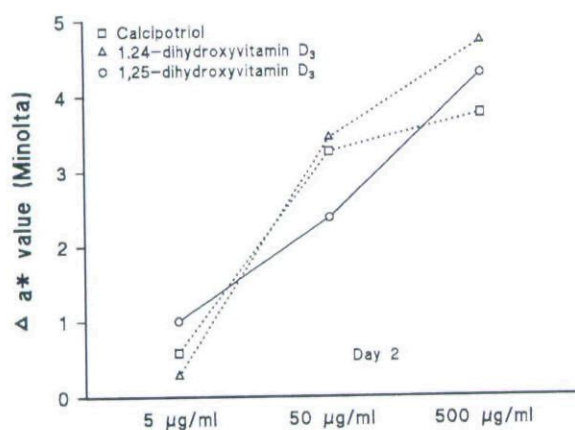


Fig. 3. Δa^* value (Minolta ChromaMeter) as a function of concentrations of substances applied ($n=8$ for each concentration).

Fig. 4. Δ skin thickness (ultrasound) as a function of concentrations of substances applied ($n=8$ for each concentration).

Results

The results of clinical scoring, measurement of cutaneous blood flow, skin colour and skin thickness on day 2 and day 3 are shown in Figs. 1–4, respectively. The Δ in the figures indicate increase compared with the value found for the placebo vehicle. A linear increase in *clinical irritation score* by

$\log(\text{concentration})$ was found for the 3 concentrations used (5, 50 and 500 $\mu\text{g/ml}$). No statistically significant difference in response was observed between any of the 3 substances. Thus, the 3 substances showed a dose-dependent and equal increase in irritancy. The results of measurement of *skin colour* and *skin perfusion* are in accordance with the clinical scoring. Increase of perfusion is

Table 2a. TEWL ($\text{g/m}^2 \text{ h}$) measured by the Evaporimeter EP1[®] on day 2 (2 h after patch removal); mean $\pm$ s.d. for $n=8$ hairless guinea pigs in each group

Test group	Placebo vehicle	MC 903 (calcipotriol)	1 α ,24(OH) ₂ D ₃ (tacalcitol)	1 α ,25(OH) ₂ D ₃ (calcitriol)
Group 1 (500 $\mu\text{g/ml}$)	7 $\pm$ 3	5 $\pm$ 2	6 $\pm$ 2	6 $\pm$ 2
Group 2 (50 $\mu\text{g/ml}$)	7 $\pm$ 1	8 $\pm$ 2	7 $\pm$ 1	6 $\pm$ 1
Group 3 (5 $\mu\text{g/ml}$)	6 $\pm$ 3	6 $\pm$ 2	6 $\pm$ 2	7 $\pm$ 3

Table 2b. TEWL ($\text{g/m}^2 \text{ h}$) measured by the Evaporimeter EP1[®] on day 3; mean $\pm$ s.d. for $n=8$ hairless guinea in each test group

Test group	Placebo vehicle	MC 903 (calcipotriol)	1 α ,24(OH) ₂ D ₃ (tacalcitol)	1 α ,25(OH) ₂ D ₃ (calcitriol)
Group 1 (500 $\mu\text{g/ml}$)	4 $\pm$ 3	3 $\pm$ 3	3 $\pm$ 2	1 $\pm$ 1
Group 2 (50 $\mu\text{g/ml}$)	4 $\pm$ 1	5 $\pm$ 2	5 $\pm$ 2	5 $\pm$ 2
Group 3 (5 $\mu\text{g/ml}$)	4 $\pm$ 2	5 $\pm$ 2	5 $\pm$ 3	5 $\pm$ 2

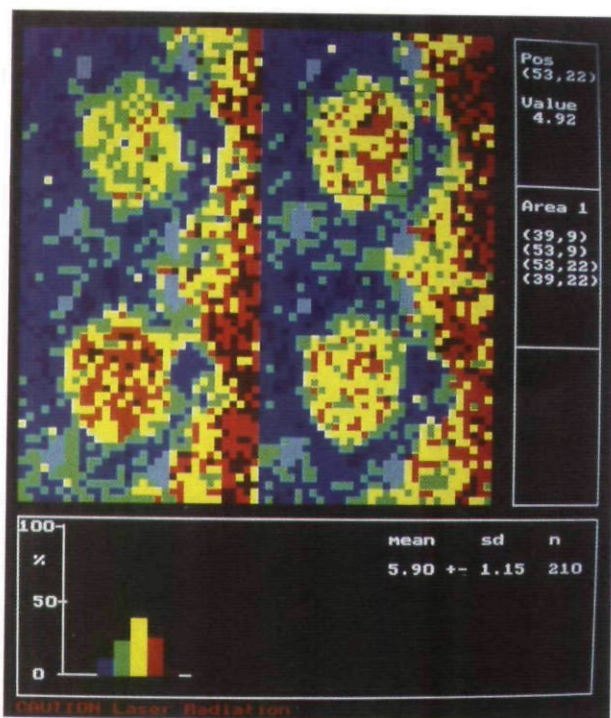


Fig. 5. 4 sequential laser Doppler image scans of a test site exposed to 500 $\mu\text{g/ml}$ of 1,25(OH) $_2\text{D}_3$. Scans were taken at day 2. For statistical evaluation a region of interest is selected in the image. LDI mean blood perfusion value $\pm$ SD within this region is shown in the figure.

illustrated in Fig. 5, which shows 4 sequential laser Doppler image scans of a test site exposed to 500 $\mu\text{g/ml}$ of 1,25(OH) $_2\text{D}_3$. Note the circular hyperperfused area in the centre of each scan corresponding to the site of application.

Based on *ultrasound scanning*, only minor edema could be found after testing with the 2 lower concentrations, while extensive edema formation was seen at the highest dose level tested. Fig. 6 represents an example of a cross-sectional ultrasound image of a test site exposed to 500 $\mu\text{g/ml}$ of 1,25(OH) $_2\text{D}_3$. The echolucent area in the dermis is caused by edema formation.

In Tables 2a, b the results of measurement of *transepidermal water loss* are given. This parameter was not affected by the substances either on day 2 or on day 3. Thus the TEWL values were normal within the concentration range tested.

Epidermal thickness measured on histological slides taken at day 3 are shown in Fig. 7, in which the % increase compared with the thickness measured for epidermis treated with placebo vehicle is shown. Epidermal thickness was found to increase in a dose-dependent way. The epidermal thickness was about 200% of the placebo control for the highest concentration i.e., 500 $\mu\text{g/ml}$.

Discussion

In the present study, the irritant potential of calcipotriol, 1 α ,24-dihydroxyvitamin D $_3$ and 1 α ,25-dihydroxyvitamin D $_3$ was compared in a hairless guinea pig model. The evaluation of irritancy was based on a multiple parameter assessment of irritancy, using both subjective clinical scoring and a number of relevant and objective non-invasive measuring techniques.

The results obtained show that the cutaneous reaction is dominated by vasodilation and increase in cutaneous blood flow, while oedema formation

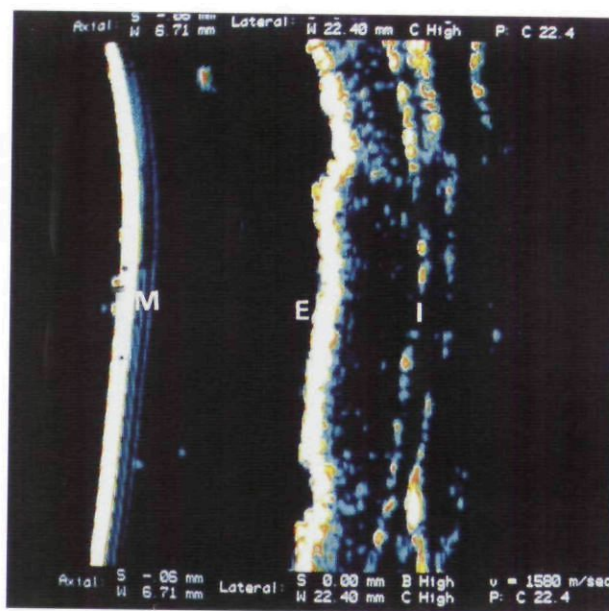


Fig. 6. Cross-sectional ultrasound image (vertically oriented) of a test site exposed to 500 $\mu\text{g/ml}$ of 1,25(OH) $_2\text{D}_3$. Image taken at day 2. M: probe membrane; E: epidermal surface; I: interface between dermis and subdermal tissue.

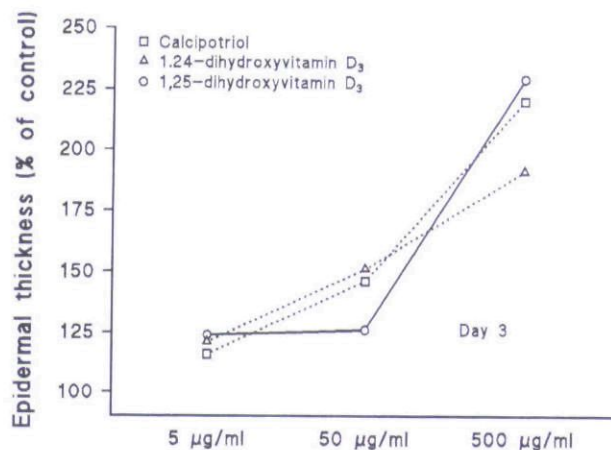


Fig. 7. Epidermal thickness (% of control) as a function of concentrations of substances applied ($n=8$ for each concentration).

is only observed in advanced reactions. The effect on skin colour and blood flow observed in the present study is in close accordance with the results of a clinical patch test study with calcipotriol and $1\alpha,25(\text{OH})_2\text{D}_3$ performed by us (15).

Evaporimetry is a method for measurement of transepidermal water loss (TEWL). TEWL has been used in the assessment of damage to the skin water barrier caused by corrosive irritants such as detergents (13). This parameter was not affected by the vitamin D analogues either on day 2 or on day 3, since TEWL values were normal in the full concentration range (5–500 $\mu\text{g}/\text{ml}$) tested. Thus, the vitamin D analogues do not affect the skin barrier properties, but cause only mild and non-caustic reactions. This special profile of irritancy again closely corresponds to our recent results obtained with calcitriol and calcipotriol in a clinical patch test study in 42 healthy volunteers (15). In this study it was additionally found that the vitamin D analogues, in contrast to an irritant like sodium lauryl sulfate (SLS), do not induce skin water barrier damage as a part of their irritancy mechanism (15). Increased TEWL has been reported after patch testing 12 subjects with calcipotriol (16). This patch test study was done with ointment formulations of calcipotriol. The ointment vehicle itself has, however, been found to increase TEWL and corrosive irritation elicited by the vehicle may easily explain this finding (17).

In the present study, test evaluations were performed both on days 2 and 3. For all parameters, the irritancy response was less pronounced on day 3 compared with day 2 recordings. The irritant effect of the 3 substances was similar on both days and for the 3 concentrations considered. Thus the study demonstrates that the irritation is transient, with reduction in all parameters from day 2 to day 3. However, clinical scores made a minor exception. The high value on day 2 for the 500 $\mu\text{g}/\text{ml}$ solution did not decrease on day 3, as the values obtained for the 2 lower concentrations did.

Epidermal thickness as an index of irritancy has previously been used in the assessment of anti-inflammatory effect of topically applied agents (18, 19) and for the assessment of irritancy of different substances (20). Measurement of epidermal thickness showed that the D-vitamin analogues in a dose-dependent way gave rise to a marked increase in epidermal thickness due to induction of epidermal cell proliferation (hyperplasia). Thus induction of hyperplasia is a part of the irritancy response of the vitamin D analogues, parallel to many other local irritants but in contrast to what is observed when the D-vitamins are used in the treatment of psoriasis. Stimulation of keratinocytes and basal cells with hyperproliferation and

hyperplasia resulting from irritation has been suggested to be a part of a repair mechanism (21, 22). Little is known about this regulatory process. Hyperplasia of human skin induced by ultraviolet radiation and retinoid application is associated with increase of acid phosphatase. This increase has been studied as a test for weak irritancy (23, 24). Mild irritation of rat skin results in an increase of acid phosphatase coinciding with acanthosis and thickening of stratum granulosum (25).

In conclusion, this study demonstrates that calcipotriol, $1\alpha,24(\text{OH})_2\text{D}_3$ and $1\alpha,25(\text{OH})_2\text{D}_3$ are equally irritant when applied in similar concentrations. A dose-dependent and parallel increase in clinical irritation score was found for the 3 substances. The irritant reaction was dominated by vasodilation and increased blood flow, while no influence on skin water barrier was found. The vitamin D analogues induced epidermal hyperplasia as a part of their irritancy mechanism, while increase in total skin thickness due to edema formation was only observed in advanced reactions. The hairless guinea pig model with multiparametric assessment to test irritancy to D-vitamins appear to be a valid model, because a similar profile of D-vitamin irritancy was previously found in humans for 2 of the compounds investigated.

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