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Effect of application time of betamethasone-17-valerate 0.1% cream on skin blanching and stratum corneum drug concentration

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Abstract

The effect of application time on skin blanching response and stratum corneum concentration after topical application of 0.1% betamethasone-17-valerate cream on healthy volunteers was assessed. Total corticosteroid content in the stratum corneum (SC) was determined at different application times (0.5, 8, 10 and 24 h) from five subjects in whom blanching was also evaluated at the same application times by visual score, colorimetry (L-, a- and b-values) and Laser Doppler flowmetry. No significant differences between corticosteroid concentration in the SC at 8, 10 and 24 h was observed when compared using ANOVA and t-test ($P > 0.05$) while drug content at 0.5 h was significantly lower ($P < 0.05$). Significant differences between the blanching response at 0.5 h and the other time points (8, 10 and 24 h) were observed by visual assessment and a-value readings from a chromameter. However, at 24 h visual score and Δa-values were lower than those measured at 8 and 10 h. but this difference was significant only for Δa-values. This findings suggest that skin blanching may not be related to drug concentration in the stratum corneum and that Δa-readings may be more sensitive and accurate than visual score in evaluating skin blanching. L-values were not significantly different at all the time points while b-values at 0.5 h were significantly different only from those at 8 and 10 h. Skin blanching was not observed when laser Doppler flow was measured while the concentration parameter was capable of detecting blanching; however, the concentration values were not significantly different at all the application times. The results of this study suggest that the duration of application should be carefully chosen to assess betamethasone-17- valerate topical bioavailability since after long application time skin blanching response may not be related to drug concentration in the stratum corneum.

Keywords: Corticosteroids; Skin blanching; Stratum corneum concentration; Filter colorimetry; Duration of application

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1. Introduction

The potency and bioavailability of topically applied corticosteroids in vivo is generally assessed by the vasoconstrictor test, i.e. skin blanching. The blanching effect is an expression of the pharmacologic activity of topically applied corticosteroids that relates well with their clinical efficacy (McKenzie and Stoughton, 1962; Cornell and Stoughton, 1985) and that can be used for measuring their bioequivalence and local bioavailability.

Studies on corticosteroids indicated that drug content in the stratum corneum could be related to the skin blanching response and that drug concentration may be used to estimate product bioavailability (Pershing et al., 1992a). Stratum corneum drug concentration could be affected by different factors among which application conditions (application time, vehicle composition, drug concentration in the vehicle, anatomical site) may play an important role (Rougier and Lotte, 1993). Many authors (Barry, 1983; Pershing et al., 1992a), studying corticosteroid topical bioavailability, reported evaluations of skin blanching and drug stratum corneum content after a single application time. Although it has been suggested that the duration of application may influence skin blanching response of topical corticosteroids (Queille-Roussel, 1988), to date little work has been carried out to assess the effect of this factor on corticosteroid topical bioavailability. The aim of the present study was to investigate the influence of application time on drug stratum corneum content and skin blanching response after topical application of 0.1% betamethasone-17-valerate cream. Therefore, total corticosteroids were determined in stratum corneum (SC) after different application times from subjects in whom blanching was also assessed at the same application time. Skin blanching is generally evaluated by visual scoring (Smith et al., 1993). However, visual skin blanching assessment lacks of reliability and strict reproducibility because of the subjective interpretation of skin blanching by the people evaluating this parameter. Furthermore, environmental conditions, such as lighting, humidity, temperature, position of the site treated, could affect the interpretation of skin blanching. The need for objective and quantitative techniques to evaluate topical corticosteroids bioavailability and/or bioequivalence in humans has led to the development of different methods for designating objectively skin blanching such as laser Doppler velocimetry or flowmetry and filter colorimetry. Recently, Queille-Roussel et al. (1991) have demonstrated the utility of readings from colorimetry in ranking the potency of different corticosteroids after their topical application. Others (Pershing et al., 1992b) used three different methods to assess betamethasone dipropionate topical bioavailability in humans from different commercial formulations: tape stripping, to determine drug content in the SC, visual score and filter colorimetry both for designating the skin blanching effect. They found that the colorimetric method provided results with less variability than visual score or the tape-stipping method. Furthermore, they observed a moderate correlation between tape stripping results and visual skin blanching readings. However, they determined drug content in the stratum corneum and correlated the observed skin blanching at one time point only, i.e. after having topically applied the drug for 6 h.

Laser Doppler flowmetry (LDF) or velocimetry enables to measure the flow rate of the erythrocytes in the corial blood circulation, which is affected by vasoactive compounds. Conflicting results have been reported on the feasibility of using this technique for evaluating the extent of skin blanching induced by topically applied corticosteroids. Amantea et al. (1983) found that measurements of skin blood flow were not suitable for evaluating corticosteroid skin blanching while Gehring et al. (1990) reported that the effect of betamethasone valerate liposomal formulation on the corial blood circulation could be successfully monitored by LDF.

In this study skin blanching was evaluated by visual score measurements, chromameter readings, concentration and flow of laser Doppler measurements in order to assess the relationship among the results obtained using these three different techniques.

2. Materials and methods

2.1. Materials

Betamethasone-17-valerate, betamethasone and prednisone were bought from Sigma (St. Louis, MO). Betamethasone-21-valerate was a kind gift of Schering (Kenilworth, NJ). Methanol, water, dichloromethane and *n*-hexane used in the HPLC analysis were of LC grade (Merck, Darmstadt, Germany). All other reagents were of analytical grade.

2.2. Human skin model

Five healthy Caucasian female volunteers (age range: 30–50 years) were used for these studies. Only subjects free of skin diseases such as mycotic or viral infections, irritant or allergic dermatitises and who were not using any drug participated in the study. They were asked not to be exposed to sun light and not to use any substance that could have masked or changed the color of the skin. Only non pregnant women were used. They were requested not to wash or wet the treated parts and not to engage in excessive physical activity, during the study periods. The volunteers were informed about the possible risk connected with their taking part in this study and provided informed consent.

2.3. Protocol for application of formulation

The topical formulation used was a 0.1% betamethasone-17-valerate W/O emulsion (Celestoderm-V 0.1% cream, Schering Plough) and a base cream (Essex base cream, Schering Plough) having the same composition but without the active principle was used as control. The creams were applied onto the upper back. The same dose of each formulation (100 mg) was placed on 2-cm diameter treatment sites and covered by modified Hill Top chambers (Hill Top Research Inc., Cincinnati, OH) in order to obtain non-occlusive conditions. The chambers were ventilated by producing small holes through the plastic and the chambers were covered using a Gore-Tex membrane (0.2-μm pore size) (W.L. Gore and Associates Inc., Elkton, MD). We used non-occlusive conditions because: (a) they more closely mirror the use conditions; (b) many authors reported that occlusion may increase skin blanching response in human subjects treated with topical corticosteroids (Coldman et al., 1971; Ostrenga et al., 1971; Poulsen and Rorsman, 1980). For each volunteer 12 chambers, equally spaced 2 cm apart, were applied on the back and divided in four groups according with the monitoring times: 0.5, 8, 10 and 24 h.

In each group, two sites were used for the test formulations and one for the base cream (control site). These time points were chosen to provide a profile of the vasoconstrictive effect of the tested compound since the intensity of the local response produced by betamethasone-17-valerate is high between 4 and 24 h following topical application, with a maximum blanching between 8 and 12 h (Magnus et al., 1980). The chamber was removed at the end of each monitoring hour and the residual formulation on the skin surface was gently removed three times using paper tissue (Kimwipe™ tissues, Kimberly Clark, USA). Skin blanching was monitored at the drug treated sites 30 min after patch removal and the corresponding stratum corneum was finally collected by stripping as described below.

2.4. Skin blanching scoring

Skin blanching was scored by a panel of three trained observers, using a 0–4 arbitrary scale (Pershing et al., 1992a), at the 0.5-, 8-, 10-, and 24-h application times. The scale has been codified as follows: 0, no variation; 1, slight, diffuse blanching with indistinct outline; 2, more intense blanching with half of the drug treated site perimeter outlined; 3, marked blanching with a distinct outline of the drug treated skin site; 4, extreme blanching with a distinct outline of the drug treated skin site.

2.5. Filter colorimetry

A Minolta Chroma Meter model CR-200 was used for all the colorimetric measurements. The measured area was 8 mm in diameter. The optical

system of the measurement head illuminated the sample using diffuse light produced by a pulsed xenon arc lamp with a viewing angle of 0°. A total of six silicon photocells were used with a double beam feed-back system to ensure accurate and consistent measurements. Three of the photocells monitored the output of the pulsed xenon arc lamp; the other three photocells measured the light reflected by the surface of the sample.

The detected signal is converted into three coordinates (L^*, a^*, and b^*) of a three-dimensional color system recommended by CIE (Commission International de l'Eclairage). The coordinate L^* represents levels of brightness between white (+100) and black (−100). The a^*-value represents the relative chromaticity between red (+60) and green (−60). The b^* coordinate represents the balance between yellow (+60) and blue (−60). The colorimeter was calibrated to standard white plate level prior to use. Calibration was performed each time the instrument was used.

2.6. Laser Doppler flowmetry (LDF)

Laser Doppler blood flow (LDF) measurements were performed using a Laser Doppler blood flow monitor instrument MBF3D Moor (Moor Instruments Ltd., USA). This instrument used a laser radiation generated by a semiconductor laser diode operating at a wavelength of 780–820 nm and a maximum accessible power of 1.5 mW. The emitted radiation entered the skin and was reflected by stationary and moving tissue components. Stationary tissue scattered and reflected the incident radiation at the same frequency. The intensity of frequency changes caused by moving erythrocytes was detected as a measure for cutaneous blood flow and erythrocytes concentration in arbitrary units. LDF concentration signal measured the number of moving erythrocytes per unit volume of tissue while flow value was proportional to the concentration signal multiplied by the speed (the mean velocity of the red blood cells).

2.7. Glass slide stripping

The stratum corneum (SC) was removed from each site using a cyanoacrylic resin which had been dropped (single drop) on a microscope slide (3 × 1 × 1 mm; Fisher Scientific Pittsburgh). The stratum corneum was stripped by adhering the slide to the skin for about 1 min. The stripping was repeated three times in the same site using three different slides to allow the total removal of stratum corneum. A single stripping removed about five stratum corneum layers (Imokawa et al., 1991).

The SC samples were protected by placing another slide on the top of the glass strips and then wrapped all around with parafilm and kept at −20°C.

Before use, the slides were washed with chloroform:methanol (2:1). The corticosteroid was extracted from the SC samples using a solution solvent consisting of sodium acetate (0.1 M, pH 4.5): ethanol (1:1). The following steps were used for extraction:

(1) Each slide was put in a 20-ml beaker and 15 ml of solution consisting of sodium acetate 0.1 M/ethanol (1:1) were added
(2) The solution was sonicated for 40 min
(3) The solution was transferred into a 20-ml vial and 5 ml of methylene chloride were added
(4) The vial was vortexed for 1 min, then sonicated for 10 min and again revortexed for 1 min
(5) The organic phase was transferred in a 20-ml tube and the solution was evaporated under nitrogen
(6) Procedures 3–5 were repeated twice, by diluting the water phase to 20 ml adding the suitable amount of methylene chloride and transferring the organic phase into the same tube every time
(7) The last milliliter of the organic solution was transferred into a 2-ml HPLC vial and evaporated until dryness.

The vials were kept at −20°C until analysis. The slide with the stratum corneum from the first step of the extraction procedure was sonicated with 20 ml of dimethylformamide which solubilized the cyanoacrylic resin while dispersing the stratum corneum. The stratum corneum dispersion was filtered with a solvent resistant filter

(Millipore Millex, SR 0.5 μm, Millipore, USA) which retained the stratum corneum. The filters were freeze-dried for 24 h in order to remove the humidity and then weighted (Sartorius microbalance, 0.2 μg of sensitivity) in order to calculate stratum corneum weight. The weighing of the filters was performed always in the same conditions (after 24 h in freeze-dryer).

2.8. HPLC analysis

Corticosteroids were determined by the method of Kubota et al. (1994). The HPLC system consisted of a Rabbit-MP constant-flow pump (Rainin Instrument Co., Berkeley, CA), a Knauer variable wavelength UV detector (Spektralphotometer, No. 731.87, Bad Homburg, Germany) set at 240 nm and an integrator Shimadzu Chromatopac (CR 601 Kyoto, Japan). A silica gel column, Lichrosphere Si-100, 10 μm, 250 × 4 mm i.d. (Merck, Darmstadt, Germany) was used for compound separation. The mobile phase consisted of 0.1% water, 4.5% methanol and 30% dichloromethane in *n*-hexane. The flow rate was 2 ml/min. The samples from glass stripping extraction were reconstituted adding 100 μl of mobile phase containing the internal standard (prednisone, 165 μg/ml) and injected into the chromatograph. The retention times for betamethasone-21-valerate, betamethasone-17-valerate, prednisone (internal standard) and betamethasone were respectively: 3.2. 3.9, 7.7, 11.4 min. The detection limit was 5 ng per sample (signal-to-noise ratio 4:1) for all the measured compounds. Recovery of corticosteroids from the glass extracts was determined by spiking stratum corneum or cyanoacrylic resin with known amount of each corticosteroid. Recovery was for stratum corneum and cyanoacrylic resin was 94 ± 5% and 96 ± 3%, respectively.

3. Results

3.1. Glass slide stripping

Topical application of 0.1% of betamethasone-17-valerate cream on human back resulted in significantly greater ($P = 0.001$) amounts of drug in the glass strippings collected from the treated sites than from control sites. SC drug content was significantly lower ($P < 0.05$) at 0.5 h compared to the other time points while no significant differences among corticosteroid concentrations in the SC were observed at 8, 10 and 24 h using ANOVA and *t*-test ($P > 0.05$) (Fig. 1).

Using the glass slide stripping method, the amount of stratum corneum removed from the sites of a particular subject was almost constant at each time point and the mean stratum corneum weight removed with three glass strips of a 2-cm diameter from all the subjects after topical application of betamethasone-17-valerate cream was not statistically different ($P > 0.05$). As shown in Fig. 1, no significant difference ($P > 0.05$) was observed when corticosteroid amounts determined by normalizing drug content in the glass-stripped SC samples with the treated surface area were compared to that obtained by normalizing drug content with the total weight of SC removed. So, all the correlations among SC drug content and

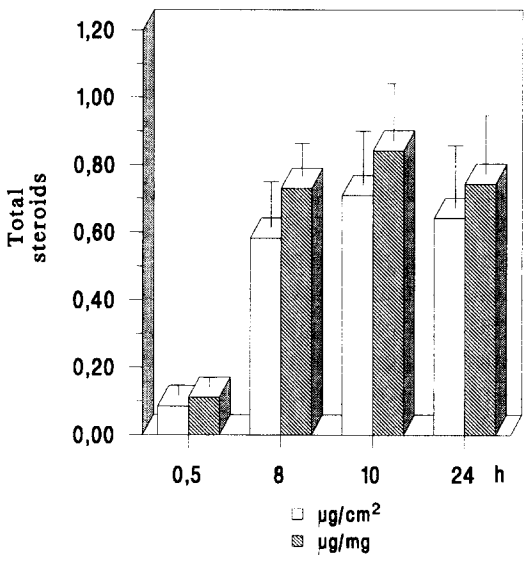

Fig. 1. Levels of coritosteroid in stratum corneum expressed as μg/cm² and μg/mg of stratum corneum.

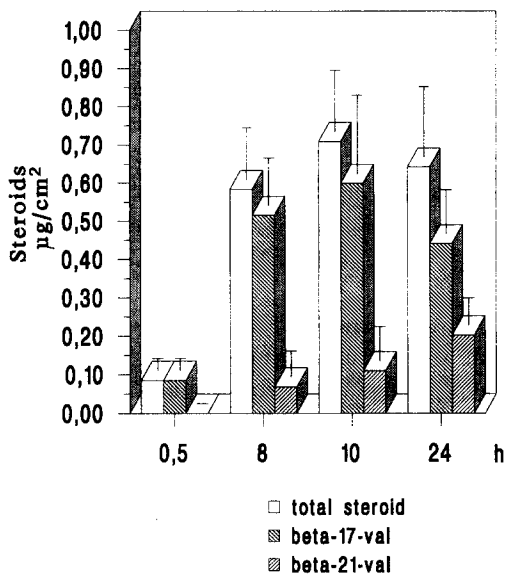

Fig. 2. Amount of total corticosteroid, betamethasone-17-valerate (beta-17-val) and betamethasone-21-valerate (beta-21-val) following the application of betamethasone-17-valerate 0.1% cream to human skin at different time intervals.

blanching response monitored using different techniques were performed expressing SC drug content as $\mu g/cm^2$.

As reported by Cheung et al., 1985 after topical application, betamethasone-17-valerate is slowly converted into an isomer, betamethasone-21-valerate which, in turn is metabolized to betamethasone. Therefore we determined both betamethasone-17- valerate and its isomer content in the SC after glass stripping and their profiles are shown in Fig. 2. The extract obtained from the stripping procedure at 30 min. after application of the cream showed only betamethasone-17-valerate. Between 8 and 24 h small amounts of betamethasone-21-valerate were detected but its level in the stratum corneum was always lower than that of betamethasone-17-valerate. Therefore this last compound accounted for majority of the total corticosteroid. When the concentration of betamethasone-21-valerate present in the SC at 0.5, 8, 10 and 24 h were compared, the data showed that the highest concentration of betamethasone-21-valerate was found at 24 h.

3.2. Skin blanching scoring

Topical 0.1% betamethasone-17-valerate cream application produced significantly greater skin blanching visual readings ($P < 0.05$) as compared with control sites (Fig. 3). There were significant differences between the extent of blanching at 0.5 h compared to the other time points ($P < 0.05$ for all the comparisons). However, the comparisons between 8 h and 10 h, 8 h and 24 h, and 10 and 24 h demonstrated no significant differences ($P > 0.05$) in the extent of blanching. Comparing visual score values from the different subjects studied (inter-subject variability) no significant difference was noted ($P = 0.664$).

3.3. Filter colorimetry

In order to compare skin color variations induced by the corticosteroid and the base cream, colorimetric values were adjusted to their own baseline values measured before applying the for-

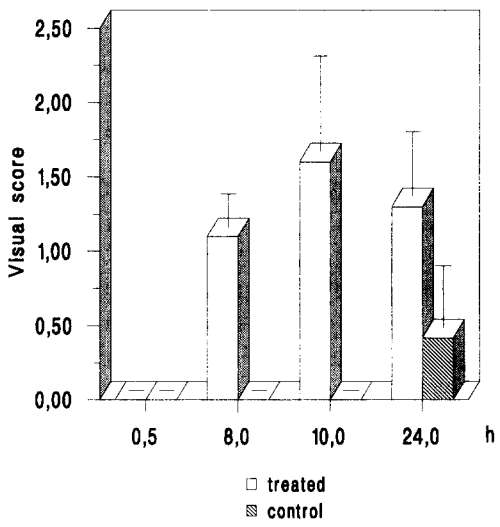

Fig. 3. Visual skin blanching scores on betamethasone-17-valerate treated sites compared to base cream treated sites (control).

mulations tested. Therefore the final values reported are the adjusted values. Results from untreated sites showed no significant differences in baseline values between the various sites on the back. Analysis of variance performed at each hour showed there were significant correlations for a- ($P > 0.05$), L- ($P > 0.05$) and b- ($P > 0.05$) values among sites within an individual subject (intra-subject variation). On the contrary, there were significant differences among the subjects (inter-subject variation). Calculations of inter-subject variations with F-statistics showed P-values of 0.00, $F = 45.57$, $P = 0.00$, $F = 101.00$, and $P = 0.00$, $F = 130.86$ for the a, b, and L parameters, respectively, indicating that these values differed significantly within the subjects studied. Therefore further studies might be conducted to investigate if this variability is duplicated in a larger population. However, it should be noted that there were not significant differences between the a- ($P = 0.773$), b- ($P = 0.794$) and L- ($P = 0.069$) values obtained from the different subjects studied for the treated sites. In order to quantify all the blanching within 24 h following non-occluded application, AUC-values were calculated plotting the variation of each parameter (L, a, b) vs. time for each subject. Mean AUC-values for each parameter obtained for treated and control sites are reported in Fig. 4. L-, a- and b-values were able to discriminate between betamethasone-17-valerate cream sites and control sites (base cream) since a significant difference was observed comparing L, a and b AUC-values obtained for the treated sites with the corresponding values observed for the base cream sites ($P < 0.05$ for all the comparisons). The pattern of skin blanching measured using L, a and b parameters was different. As regards Δa-values (Fig. 5), there were significant differences ($P < 0.05$) between the extent of blanching observed at 0.5 h and that measured at all the following time points while there were no significant difference comparing the values at 8 and 10 h. It is worthwhile noting that Δa-value at 24 h was significantly lower compared to those at 8 and 10 h. Comparing L-values at all the time points no significant difference was observed while for b-values the only significant difference was observed comparing the values at 0.5, with those at 8 and 10 h (data not shown).

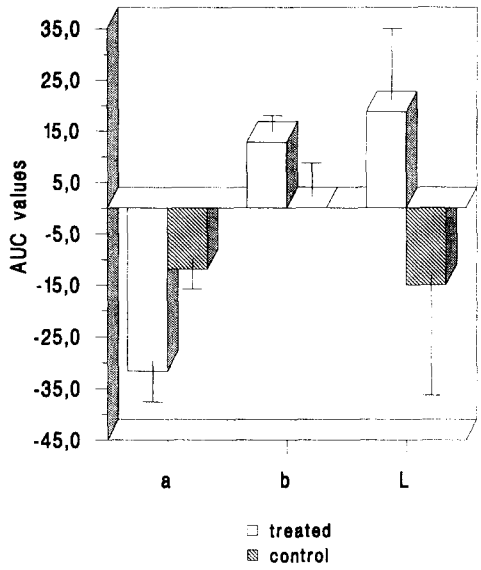

Fig. 4. Mean AUC values for L-, a-, and b-values for drug and base cream (control) treated sites.

3.4. Laser Doppler flowmetry

Quantification of all the skin blanching within 24 h following non- occluded application was performed calculating AUC-values by plotting the

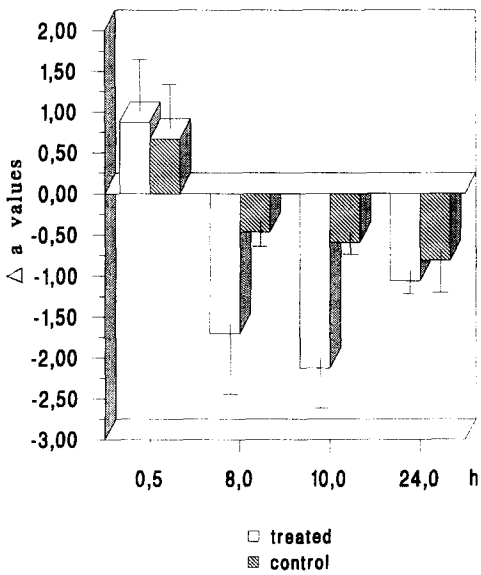

Fig. 5. Profile of skin blanching recorded by Δa-values.

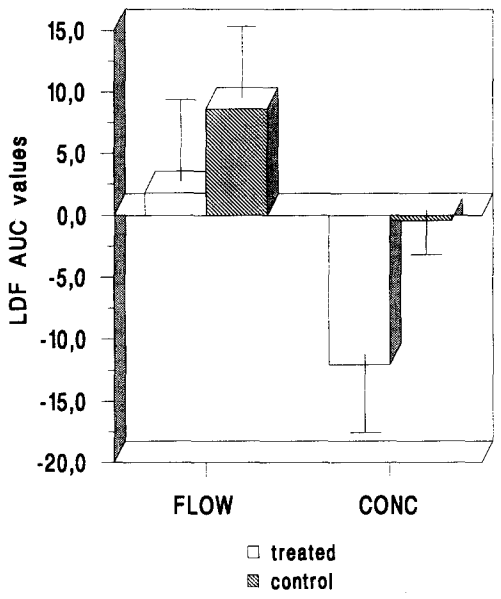

Fig. 6. Mean AUC values for laser Doppler values for drug and base cream (control) treated sites (conc. = concentration parameter).

variation of the flow and concentration parameters vs. time for each subject. Mean AUC-values for each parameter obtained for treated and control sites are reported in Fig. 6. Topical application of 0.1% betamethasone-17-valerate cream did not produce any significant blanching response ($P > 0.05$) compared to the base cream sites (control) when laser Doppler flow parameter was measured.

On the contrary, a significant difference ($P < 0.05$) was observed between treated sites and control sites determining the concentration parameter of the LDF instrument (Fig. 6). Using the concentration parameter no significant difference ($P > 0.05$) was observed among the values obtained at all the time points, apart from the values at 0.5 h which significantly differed from those at 8 h.

4. Discussion

Recently, Pershing et al. (1992a) reported that the objective analysis of drug stratum corneum content could be regarded as an useful method for evaluating topical corticosteroid bioavailability and bioequivalence. This approach could be also used to assess the effect of application conditions such as drug concentration, vehicle composition and application time on corticosteroid bioavailability. As reported by Rougier and Lotte (1993), drug concentration in the horny layer is closely related to the duration of application. Stripping of the stratum corneum is generally utilized as a technique for determining drug content, distribution and metabolism in the outer layers of the skin (stratum corneum). In the present study we have used an innovative technique of stripping (glass slide stripping), which has been successfully employed for quantifying SC lipids (Imokawa et al., 1991). This method seems to be more practical, less time-consuming and easier to standardize compared with the tape-stripping method more often used until now since stratum corneum is removed after only three strippings. In this work, using this technique, a similar corticosteroid concentration was found in the SC at different drug application time, namely 8, 10 and 24 h while at 0.5 h SC drug content was significantly lower. Visual and instrumental assessment (colorimetry and Laser Doppler flowmetry) did not detect any blanching response at 0.5 h of betamethasone topical application although at this hour betamethasone-17-valerate was found in the SC. These results suggest that combination of SC drug concentration and time of exposure of the skin to the drug could influence the initiation of the blanching response. Thus, although corticosteroid was observed in the SC by 0.5 h, the drug uptake into SC and the time of skin exposure to the drug were not sufficient to produce blanching. At 8, 10 and 24 h both visual and instrumental readings, apart from LDF flow parameter, recorded a skin blanching response. Other authors (Amantea et al., 1983), studying in vivo effect of topically applied corticosteroids, reported that laser Doppler flowmetry was not able to monitor induced skin blanching. In this study we determined LDF concentration values, in addition to flow, to assess if this parameter could provide better results. LDF concentration values allowed us to detect skin blanching while flow values in the treated sites did not significantly

change compared to control and untreated sites. The different physiological variations measured by these two parameters could account for the conflicting results obtained in this study. Since, in some cases, a vasoactive compound could increase red blood cell concentration while decreasing the speed and vice versa the resulting LDF flow values could not change. So, vasodilation or vasoconstriction could occur even if constant LDF flow values are recorded. However, it should be noted that LDF concentration parameter did not properly discriminate the skin blanching response after different application times of 0.1% betamethasone-17-valerate cream and therefore this technique could be regarded as less sensitive than visual and colorimetry assessment. As shown in Figs. 3 and 5, a similar profile of skin blanching response was observed by visual assessment and Δa readings from the chromameter. However, it should be noted that at 24 h Δa-values were significant lower than those at 8 and 10 h and no significant difference was observed between treated and control (base cream treated sites). Visual score at 24 h was also lower but not significantly different from 8 h and 10 h. The lower skin blanching recorded at 24 h could be due to: (a) a tachyphylaxis phenomenon, as reported by other authors following long application time of topical corticosteroids (Queille-Roussel, 1988); (b) betamethasone-17-valerate metabolic conversion into betamethasone 21-valerate which decreased betamethasone-17-valerate concentration in the SC. The skin blanching response recorded at 24 h by visual score and Δa readings for base cream treated sites requires further studies to be clearly understood since it could be due to different factors such as increased skin hydration or presence of potential vasoactive components in the formulations. However, the differences in skin blanching response recorded by visual score and Δa readings suggest that instrumental assessment by Δa-values could provide a method more sensitive and accurate than visual score. Furthermore, the lower skin blanching response at 24 h could indicate that the response may be independent of the SC drug content since at 24 h betamethasone-17-valerate concentration in the SC was not significantly different from those determined at 8 h and 10 h. It is worthwhile to note that a lack of correlation between hydrocortisone penetration and the blanching effect determined by visual score in humans at different times after topical application was also reported by Caron et al. (1990).

Comparing the results obtained from visual score and chromameter readings, a strong relationship was observed comparing visual score to a value ($r = -0.950$) and to L-value ($r = 0.989$) while lower correlations were found with b-value ($r = 0.661$) (Fig. 7).

Since Pershing et al. (1992a) reported a good relationship between SC corticosteroid content and skin blanching response, we compared visual and instrumental (L, a, b,) readings with betamethasone-17-valerate content in the SC. The following rank of correlation was obtained: visual score ($r = 0.993$) > L-value ($r = 0.991$) > a-value ($r = 0.954$) > b-value ($r = 0.650$). However, in the present study these correlations are not truly significant since drug concentration in the SC was not significant different after 8, 10 and 24 h of drug application. In conclusion, the results of this study suggest that the duration of application should be carefully chosen to assess

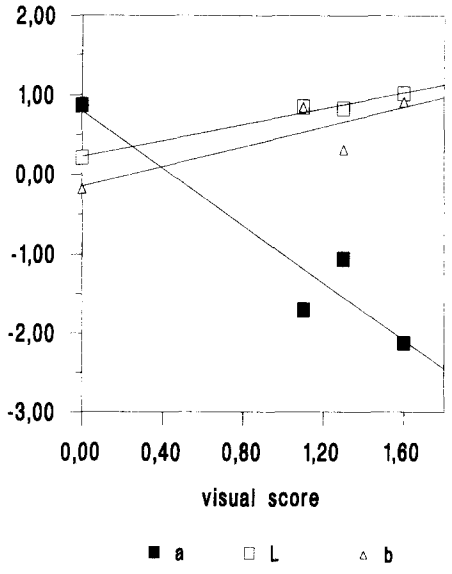

Fig. 7. Relationship between visual measurements and instrumental readings (L,a,b) at different time points.

betamethasone-17-valerate topical bioavailability since after long application time skin blanching response may not be related to drug concentration in the stratum corneum. Further studies are needed to better elucidate the relationship between the cumulative amount of drug released from the stratum corneum to the underlying viable tissue and the skin blanching induced by corticosteroids.

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