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Variability and correlation of chromameter and tape-stripping methods with the visual skin blanching assay in the quantitative assessment of topical 0.05% betamethasone dipropionate bioavailability in humans

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Summary

Bioavailability between five commercial 0.05% betamethasone diproprionate formulations in human skin is demonstrated with three methods; visual skin blanching, tape-stripping and a chromameter. Reproducibility of each method is evaluated for both intra- and inter-subject variability. The variability (coefficient of variation) in the intra-subject studies is consistently less than that in the inter-subject studies for all methods evaluated. The variability in the subjective visual skin blanching assay increases as a function of time. Variability in the tape-stripping technique performed at one time point only ranges from 7 to 30% in the intra-subject study to greater than 70% in the inter-subject study. Variability in the *a* scale on the chromamater is consistent over time (approx. 24%). The *b* scale on the chromameter demonstrates an inter-subject variation of about 15%, while the luminosity scale demonstrates a variability of approx. 6%. The rank order of 0.05% betamethasone diproprionate formulation potency is similar between the visual skin blanching assay, tape-stripping and the *a* scale on the chromameter. Further, the rank correlation between tape-stripping and the *a* scale on the chromameter with the subjective visual skin blanching assay is moderate to excellent ($r = 0.6$, and -0.9, respectively). Thus, tape-stripping and the chromameter offer the investigator two objective, standardized and noninvasive methods with which to compare bioequivalence of topical corticosteroids.

Introduction

Bioavailability differences between manufactured topical corticosteroids have historically been evaluated with a bioassay of skin blanching (McKenzie and Stoughton, 1962; Barry and Woodford, 1974, 1975; Cornell and Stoughton, 1984; Stoughton, 1987). The variability in skin blanching between subjects and laboratories with the same topical drug formulation however, can be great due to (1) biological and physicochemical differences in skin among the test population, (2) the subjective interpretation of skin blanching by the person(s) evaluating the blanching parameter,

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or (3) the environment in which the blanching response is evaluated. The biological and physicochemical differences in skin may be controlled in part by using a population of subjects which demonstrate a particular desired response to a topical corticosteroid (Meyer et al., 1988). While this lessens the variability among the tested population, it may not represent use of the drug in a clinical population. Interpretation and scoring the blanching response may also vary among investigators (McKenzie and Stoughton, 1962; Peplar et al., 1971, Gibson et al., 1984). The environmental conditions in which the skin blanching response is evaluated may also influence the interpretation of the skin blanching response due to differences in lighting, humidity, temperature, position of the skin site treated during assessment of the blanching response, etc.

The need for an objective, yet quantitative method to assess bioequivalence of topical corticosteroids in human skin in vivo led to the present study, in which two other methods, tape-stripping and a chromameter are evaluated using various formulations of topical 0.05% betamethasone diproprionate. Chemical analysis of drug content in the treated skin site via tape-stripping is a technique which has been utilized to quantitate drug disposition in skin (Schaefer et al., 1977) and more recently to predict the percutaneous absorption of topically applied compounds (Dupuis et al., 1984) as well as the pharmacodynamic activity of topical iododeoxyuridine (Sheth et al., 1987), hydrocortisone (Caron et al, 1990) and betamethasone dipropionate (Pershing et al., 1991). The Minolta™ CR-200 chromameter is a new instrument, which objectively quantitates color numerically, in terms of hue, light value, and saturation. These parameters are described in three scales; red-green color (a) and yellow-blue color (b) and in luminosity (L). Its application for ranking the potency of topical corticosteroids has been demonstrated (Queille-Roussel et al., 1991). Temporal and investigator variability in the evaluation of the blanching response is substantially reduced with this technique due to the inclusion of a control surface (ceramic plate) for standardization of the instrument with each use. Both tape-stripping and the chromameter offer a standardized, objective approach to compare bioequivalence assessment among investigators. Despite availability of these methods, they have not been evaluated for their precision and/or relevancy to the existing reference method in assessing in vivo bioequivalence, the visual skin blanching assay, as developed by McKenzie and Stoughton (1962). The present study evaluated the intra- and inter-subject variability of these methods used to compare bioequivalence of topical 0.05% betamethasone diproprionate products in human subjects and the correlation of these methods with the visual skin blanching assay.

Materials and Methods

Chemicals

The 0.05% betamethasone diproprionate ointments (Diprolene® augmented ointment; DLO and Diprosone ointment; DSO), creams (Diprolene AF® cream; DLC and Diprosone cream; DSC) and lotion (Diprosone lotion; DSL) tested are all manufactured by Schering Corp. and were used as purchased from the University of Utah Hospital Pharmacy. The Minolta™ CR-200 chromameter was purchased from Applied Technical Products, Inc., Denver, CO. Degassed acetonitrile (Burdick Jackson HPLC grade) was used to extract drug from the tape-strips as purchased from Baxter, Salt Lake City, UT. Degassed double-distilled water was used as supplied in-house for preparation of the HPLC mobile phase. The 1.5 ml polypropylene microcentrifuge tubes used for extraction of the tape-strips were purchased from Intermountain Scientific, Bountiful, UT. Transpore™ tape (3M, St Paul, MN) used for tapestripping and the Dermicare™ tape (Johnson & Johnson, NJ) used to make the protective nonocclusive guard were purchased from the University of Utah Hospital Pharmacy. The 2.2 cm diameter rubber O-rings were purchased from a local plumbing supply store. The 8 mm diameter disposable punch biopsies used to prepare the tapestripping discs from the Transpore™ tape were used as purchased from Accuderm, Ft. Lauderdale, FL.

HPLC protocol

Betamethasone dipropionate was identified chromatographically (Pershing et al., 1991) at a retention time of 6.45 min by HPLC (Beckman binary gradient model 334) on a 4.6 mm × 25 mm C-18 RP column (Altex, Palo Alto, CA) at 25°C, at a rate of delivery of 1.2 ml/min of degassed acetonitrile : distilled water (65 : 35 v/v) mobile phase and a 20 μl sample loop (Pershing et al., 1991). Drug in tape-stripped stratum corneum was detected from the absorbance spectrum of betamethasone diproprionate at 254 nm UV fixed wavelength detection (Beckman model 164). Drug concentrations in the tape-stripped samples were determined from dipropionate standard curves (0.05–1 mg/ml) generated with the pure compounds, generously supplied by Schering Corp. (Kenilworth, NJ). The limit of sensitivity for betamethasone dipropionate was 0.05 μg/ml or 1 ng on column. The standard curves were linear for betamethasone dipropionate ($r = 0.9993 \pm 0.0015$; mean ± SD for $n = 8$) with an interrun precision (coefficient of variation) of 0.15%.

The first tape-strip collected was discarded due to potential drug remaining on the skin surface. The remaining 10 tape-strips collected were combined in a 1.5 ml polypropylene microcentrifuge tube, capped and extracted with 250 μl of acetonitrile by vortexing at high speed for 1 min. The microcentrifuge tubes were then centrifuged at 5000 rpm for 8 min (Biofuge A, Heraeus, Germany) and the resulting supernatant was transferred to a clean labeled microcentrifuge tube and injected onto a 20 μl loop on the HPLC. A single extraction of the tape-strips containing drug and stratum corneum was sufficient for the routine recovery of more than 90% of the drug. Drug concentrations were calculated against known pure drug levels. Chromatography of the tape + stratum corneum vs tape-only did not reveal any interfering peaks with betamethasone dipropionate.

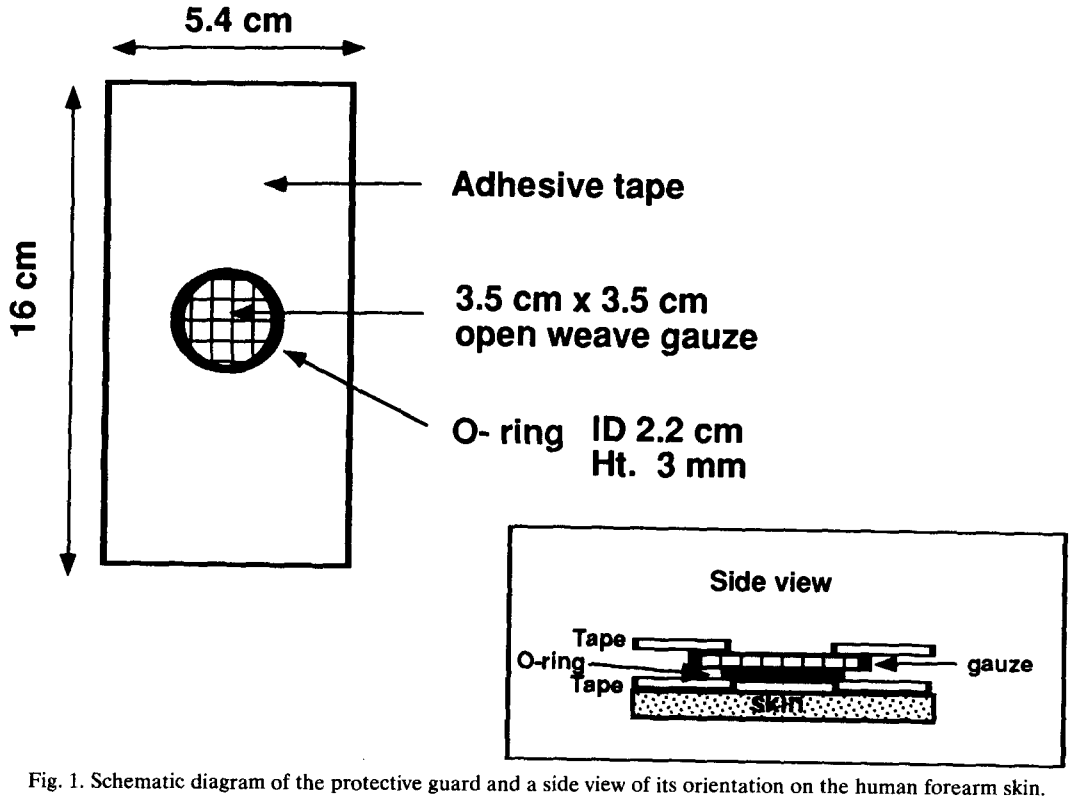

Fig. 1. Schematic diagram of the protective guard and a side view of its orientation on the human forearm skin.

Nonoccluding protective tape guard

The treated skin sites were protected from the environment with a nonoccluding tape guard (Fig. 1), consisting of a 2.2 cm internal diameter rubber. O-ring (3 mm thick) covered with a 3.5×3.5 cm piece of single-ply open-weave cotton gauze (Johnson & Johnson, U.S.A.) and attached over a 2.0 cm diameter hole in the midsection of a 5.4×16 cm piece of Dermicare™ tape (Johnson & Johnson, U.S.A.). This protective guard allowed air circulation to the treated skin without occlusion.

Human subjects in the test population

Three human volunteers, one male and two females, ages 19–38 years, were used in the intra-subject variability study. Six female and four male human volunteers, between the ages of 19 and 50, were used in the inter-subject variability studies. The volunteers were all naive to topical drug therapy and not prescreened for sun exposure, but their skin pigment was unchanged over the course of the study period. All volunteers were prescreened in advance for a +2 or greater skin blanching response to 6 h of nonoccluded treatment with 0.1% triamcinolone cream (Fougera, Melville, NY).

Intra-subject variability studies

In the intra-subject variability study, the three techniques were evaluated in three human subjects following 6 h of nonoccluded but protected treatment with 0.05% Diprolene® augmented ointment (Schering Corp., Kenilworth, NJ) on the ventral forearm. Five sites, each 2.2 cm diameter, were demarked 2 cm apart on the ventral forearms of three human subjects, being careful to apply the formulations 6 cm below the antecubital fossa and 6 cm above the wrist. Four sites on each forearm were treated with 0.05% Diprolene® augmented ointment (2.6 μl formulation/cm^2 surface area) and one site served as the untreated control skin site. All sites were protected from the environment with a nonoccluding tape guard. After 6 h of drug treatment, the protective guards were removed and residual drug at the treated sites was removed three times with Kimwipe™ tissues (Kimberley-Clark, U.S.A.). Untreated control sites were also wiped three times with the tissues. The treated skin sites on the left arm were evaluated visually for skin blanching by a single observer, according to a skin blanching scale from 0 (no blanching) to 4 (maximal blanching) (Pershing et al., 1991) and on the *a*, *b* and luminosity scales of the chromameter at 6, 8, 16, 18, 20 and 24 h after drug application. The skin sites on the right arm were tape-stripped 11 times over the same skin area with 8 mm diameter pieces of Transpore™ tape (3M, St Paul, MN) within 1 h of residual drug removal. All treated skin sites were evaluated for skin blanching and tape-stripped by a single investigator. The smaller tape-strip diameter was used to avoid edge effects in drug deposition on the 2.2 cm diameter treated skin surface and thus reduce artifactually high drug concentrations in the analysis. The first tape-strip was discarded on the basis of potential surface drug contamination and the subsequent 10 tape-strips were combined into a clean, labeled 1.5 ml polypropylene microcentrifuge tube and submitted to drug extraction and analysis on HPLC.

Inter-subject variability studies

Determination of the inter-subject variability in quantitating bioavailability of five formulations of 0.05% betamethasone dipropionate with the three above techniques was performed in 10 human volunteers. Five sites, each 2.2 cm diameter, were demarked 2 cm apart on both ventral forearms of 10 human subjects. All treatments were placed within the skin area that was 6 cm below the antecubital fossa and 6 cm above the wrist. Each ventral forearm received a single 2.6 $\mu l/cm^2$ dose of each formulation, applied in a latin square blinded fashion for 6 h. Another untreated skin site served as the untreated skin control site. All sites were protected from the environment with a nonoccluding, but protective tape guard. At the end of 6 h, the protective guards were removed and residual drug at the treated and control skin sites removed three times with Kimwipe™ tissues (Kimberley-Clark, U.S.A.). The treated skin sites on the left arm were then evaluated by a single observer for visual skin blanching, and the *a*, *b* and luminosity scales on the chromameter,

TABLE 1

Intra-subject [a] variability to topical 0.05% Diprolene® augmented ointment

Time (h) [c]	CV (%)														
	Visual skin blanch			a scale [b]			b scale [b]			L scale [b]			μg in 10 Tape strips		
Subject:	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
0	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0	0.0
6	0	0	27.8	9.7	27.3	13.2	4.7	5.5	6.3	0.8	0.7	2.0	8.2	24.4	46.7
8	0	0	14.7	3.8	26.2	30.8	6.0	2.4	8.7	0.9	1.7	2.0			
16	0	0	18.0	5.9	18.7	27.4	5.1	2.4	5.5	1.4	0.9	1.8			
18	23.1	8.6	29.2	5.9	14.3	21.9	5.3	4.1	5.8	0.7	0.6	1.7			
20	23.1	20.8	36.8	3.1	4.8	21.2	3.6	3.4	9.0	0.5	0.9	1.9			
24	0	0	36.8	4.0	9.3	26.1	3.0	6.6	7.1	1.9	0.7	2.8			
32	38.5	96.2	40.8	7.1	9.5	6.9	5.0	6.2	5.6	0.8	0.6	1.0			
48	200	0	250.0	1.4	7.9	20.4	6.4	7.0	5.4	1.0	0.8	2.1			

[a] $n = 3$ human volunteers, with five sites treated per individual.

[b] Minolta™ CR-200 chromameter.

[c] Time (h) after drug application. Drug treatment stopped at 6 h.

TABLE 2

Inter-subject [a] variability to 0.05% betamethasone dipropionate formulations

Time (h) [c]	CV (%)																									
	Visual skin blanching					μg in 10 tape-strips					*a* scale [b]					*b* scale [b]					*L* scale [b]				
	DSL [d]	DSC [d]	DLC [d]	DSO [d]	DLO [d]	DSL	DSC	DLC	DSO	DLO	DSL	DSC	DLC	DSO	DLO	DSL	DSC	DLC	DSO	DLO	DSL	DSC	DLC	DSO	DLO
0	0	0	0	0	0	0.0	0.0	0.0	0.0	0.0	14.8	14.8	14.8	14.8	14.8	13.7	13.7	13.7	13.7	13.7	5.3	5.3	5.3	5.3	5.3
6	47.0	35.9	65.0	76.3	35.5	77.6	79.1	99.0	98.0	90.0	20.1	17.8	18.2	20.3	31.1	11.8	15.4	17.0	17.0	12.9	5.9	7.0	7.2	6.4	5.7
8	24.7	21.1	49.5	65.0	14.3						19.6	11.8	20.3	25.0	24.3	12.4	14.2	17.1	14.7	15.0	5.3	6.3	6.9	6.4	4.7
16	24.7	29.4	43.2	57.9	38.6						21.0	19.9	20.4	19.1	25.9	11.3	12.2	15.9	12.5	14.5	5.5	6.3	6.3	5.8	4.5
18	24.7	63.1	51.1	43.7	52.4						18.8	22.1	20.6	17.1	26.9	12.2	13.6	17.8	14.7	14.1	5.3	6.6	5.9	5.0	5.1
20	46.1	64.8	89.2	63.3	84.4						17.8	18.9	18.1	15.5	21.0	12.0	12.9	18.2	14.9	14.7	5.1	6.2	5.4	4.8	5.4
24	57.4	100.1	86.1	79.1	78.6						17.4	20.2	14.8	35.2	20.6	12.2	13.9	19.1	15.6	16.0	5.4	6.9	5.8	5.1	5.3

[a] $n = 10$ human volunteers.

[b] Minolta™ CR-200 chromameter.

[c] Time (h) after drug applied to skin site.

[d] Formulations tested: DSL, 0.05% Diprosone Lotion; DSC, 0.05% Diprosone cream; DLC, 0.05% Diprolene AF® cream; DSO, 0.05% Diprosone ointment; DSO, 0.05% Diprolene® augmented ointment.

as described above. The skin sites on the right arm were tape-stripped 11 times by a single investigator and submitted to HPLC analysis, as described above.

Statistics

Statistical analyses of the data were performed with Statview 512™ software from BrainPower, Inc., Calabasas, CA. Intra-class variation in the intra-subject study was statistically analyzed at 95% confidence with a single factor, repeated measures ANOVA. Fisher PLSD analysis of variance (Tukey's ANOVA) was used to determine statistical significance between the five formulations in the inter-subject study. Correlation between the mean visual skin blanching response and the mean response of the tape-stripping and chromameter methods was analyzed with the nonparametric Spearman's rank correlation coefficient.

Results

The intra-subject coefficient of variation of the visual skin blanching response produced by 0.05% Diprolene® augmented ointment (DLO) applied to four sites on the same arm increased over time from 0 to 35% (Table 1). The coefficient of variation of drug content in the tape-stripped stratum corneum was in the range of 8–47% in the three subjects. The average coefficient of variation of *a*, *b* and luminosity scales on the chromameter over all time points fell within the ranges of 5–32, 4–7 and 1–3%, respectively, in the three individual subjects. The variability in all three parameters on the chromameter was less than that of the visual skin blanching or tape-stripping methods. The variability in these parameters on the same anatomical location in each individual was as low as 1% in some individuals or as great as 34%, depending on the parameter evaluated. The lowest coefficient of variation among all parameters evaluated occurred 8 h after drug application. The range in the variability observed with all techniques demonstrated that even in the same human skin source there was a low but inherent variability (up to 35%) of drug uptake into human stratum corneum and pharmacodynamic response (skin blanching) to topical corticosteroids. Intra-class correlation of the four treated skin sites within a particular subject was low, thus within an individual, the skin response to DLO was similar and independent of the position of the topical formulation on the subject's forearm.

Whether the variability in the skin response to topical 0.05% betamethasone dipropionate in this small test population represented the variability of a larger population was evaluated with a latin square application of 0.05% DLO to the ventral forearms of 10 naive human subjects. With a larger population of human subjects ($n = 10$), the coefficient of variation in the visual skin blanching score following application of 0.05% DLO was the lowest (12%) at 8 h, thereafter increasing in a time-dependent manner to approx. 80% at 20–24 h after drug application (Table 2). Variability in the *a* scale on the chromameter remained relatively constant, 20–27%, over the same time periods. The variability in the *b* and luminosity scales over time was also relatively constant over time, approx. 14 and approx. 5%, respectively. The variability of drug content in tape-strips collected from the 10 subjects treated with DLO for 6 h was 90%. The variability in the skin response to DLO among 10 subjects was therefore greater with the visual skin blanching and tape-stripping methods than any of the scales on the chromameter. Further, the variability in skin response to DLO between different subjects was greater than within the same subject.

The inter-subject variability with the three methods observed with DLO was compared with other formulations of 0.05% betamethasone dipropionate; two cream formulations, DSC and DLC, a lotion formulation, DSL and another ointment formulation, DSO (Table 2). Variability in all of the evaluated parameters increased over time. The lowest variability among all methods was detected 8 h after drug application in both the intra- and inter-subject study. Thus, the 8 h time point was selected to compare and contrast the abilities of the three techniques to differentiate between formulations of 0.05% betamethasone dipropionate. Variability in the objective

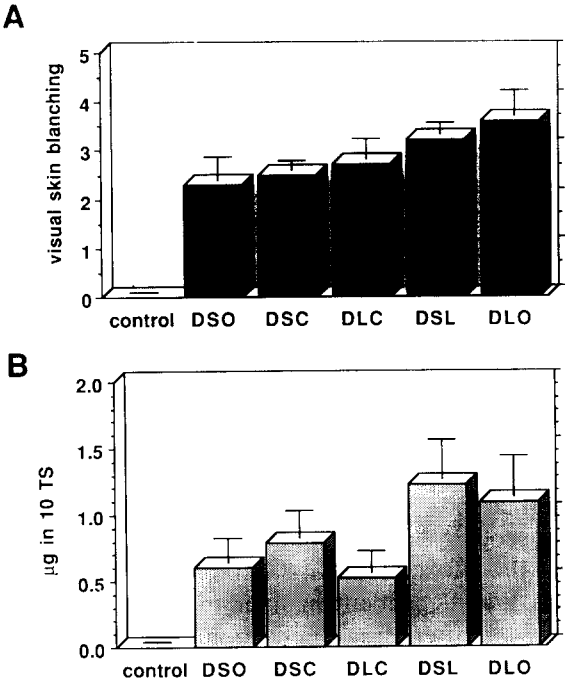

Fig. 2. Ranking of five formulations of 0.05% betamethasone dipropionate according to the (A) visual skin blanching response and (B) amount of betamethasone dipropionate in 10 tape-strips (μg in 10 TS) 8 h after drug application. Mean and 1 SE for $n = 10$ subjects. Control, untreated skin site; DSC, Diprosone cream; DLC, Diprolene AF® cream; DSO, Diprosone ointment; DSL, Diprosone lotion; DLC, Diprolene® augmented ointment.

measure of the amount of drug in the tape-stripped treated stratum corneum, and in the *a*, *b* and luminosity scales on the chromameter 8 h after drug application, was independent of formulation applied to the skin. In contrast, the variability in the subjective visual skin blanching response was 2–3-fold greater in DSO and DLC formulations than in the other formulations.

All formulations of 0.05% betamethasone dipropionate produced visual skin blanching responses at the treated skin site that were significantly different ($p < 0.05$) from the untreated control site (Fig. 2A). The DLO and DSL formulations produced statistically greater ($p < 0.05$) skin blanching response 8 h after application than those of the DLC, DSC or DSO formulations. The rank order of the skin blanching response with the five formulations was DLO > DSL > DLC > DSO ≥ DSC.

Topical application of all formulations of 0.05% betamethasone dipropionate on human forearm skin resulted in significantly greater ($p < 0.05$) amounts of drug in the tape-strips collected from the treated skin sites than the untreated control skin sites (Fig. 2B). Both DLO and DSL demonstrated significantly greater amounts of drug in the tape-strips ($p < 0.05$) than the three other formulations. No significant differences in drug content were noted between DSO, DSC or DLC. Rank correlation between

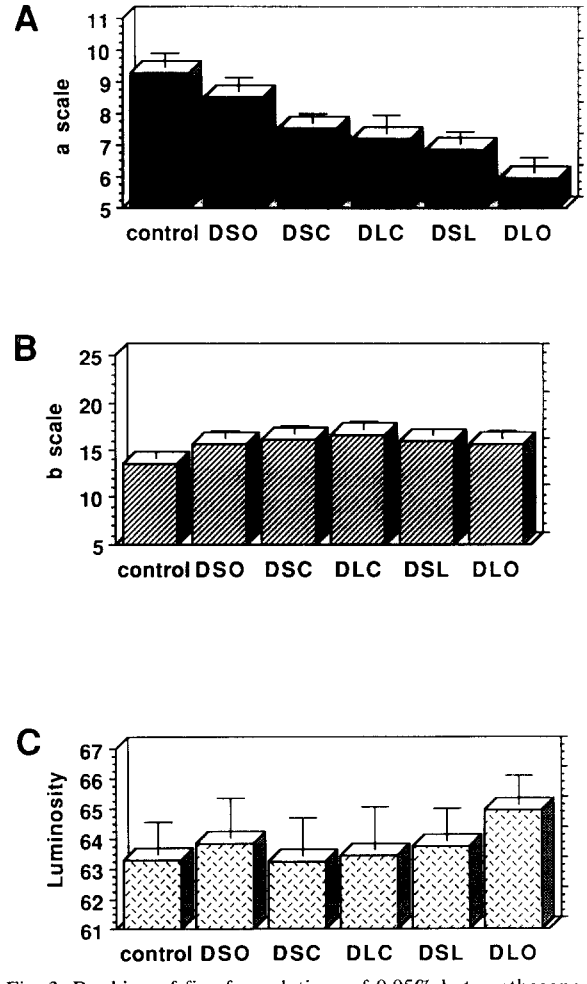

Fig. 3. Ranking of five formulations of 0.05% betamethasone dipropionate according the the response on the (A) *a* scale, (B) *b* scale, and (C) luminosity scale on the chromameter, 8 h after drug application. Mean + 1 SE for $n = 10$ subjects. Control, untreated skin site; DSC, Diprosone cream; DLC, Diprolene AF® cream; DSO, Diprosone ointment; DSL, Diprosone lotion; DLO, Diprolene® augmented ointment.

the mean skin blanching response and mean amount of drug in 10 tape-strips was moderate, $r = +0.6$, but not statistically significant ($p < 0.05$) with 10 subjects (Fig. 4D).

Differentiation among the five formulations was also demonstrated on the *a* scale of the chromameter (Fig. 3A). All formulations produced a skin response that was significantly different ($p < 0.05$) from that of the untreated control site. DLO and DSL produced changes in the treated skin site that were significantly different from those of DSC, DSO and DLC. Again, no significant differences were noted between the DSC, DSO or DLC. Formulations that produced large skin blanching values also resulted in low *a* scale values. Rank correlation between the mean skin blanching response and the mean *a* scale response in 10 subjects was excellent, $r = -0.9$ (Fig. 4C).

All topical 0.05% betamethasone dipropionate formulations produced significantly greater readings ($p < 0.5$) on the *b* scale of the chromameter as compared with the untreated control site (Fig. 3B). Despite the low variability in the *b* scale response to corticosteroid treatment among the 10 individuals, the *b* scale was unable to statistically differentiate the five formulations. Changes in the mean visual skin blanching response did not correlate with the *b* scale on the chromameter, $r = +0.1$ (Fig. 4A).

Despite the low inter-subject variability in measuring the skin response to 0.05% betamethasone dipropionate, the luminosity scale on the chromameter did not measure any statistically significant difference between drug-treated and untreated skin sites (Fig. 3C). Correlation between the mean skin blanching response and the mean luminosity response to the five formulations was poor, $r = +0.4$ (Fig. 4B).

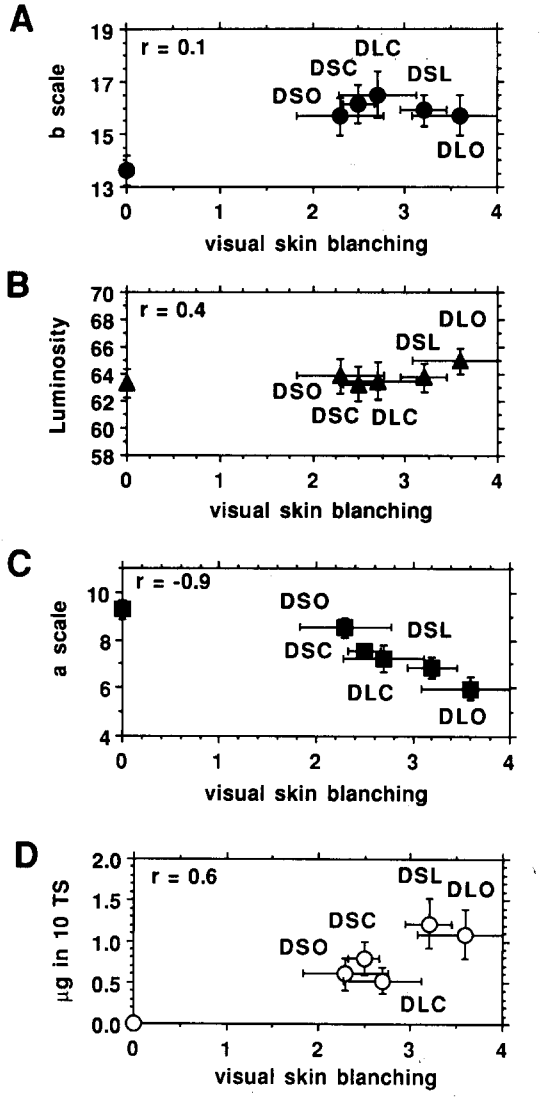

Fig. 4. Correlation between visual skin blanching and the (A) mean *b* scale on the chromameter, (B) mean luminosity scale on the chromameter, (C) the mean *a* scale on the chromameter, and (D) mean amount of betamethasone dipropionate in 10 tape-trips, 8 h after application of five formulations of 0.05% betamethasone dipropionate on the forearm of human subjects. Mean ± 1 SE for $n = 10$ subjects. DSC, Diprosone cream; DLC, Diprolene AF® cream; DSO, Diprosone ointment; DSL, Diprosone lotion; DLO, Diprolene® augmented ointment.

Discussion

As expected, the variability between human subjects (inter-subject) was greater than in the same subject (intra-subject) and is in agreement with those observations of intra and inter-human skin variability on the human skin sandwich flap (Pershing et al., 1987). The variability measured with all three techniques; tape-stripping, visual skin blanching and the chromameter, likely reflects the differences in both drug uptake into the stratum corneum and corticosteroid receptor dynamics over time as influenced by the local physi-

ology, physicochemistry and biochemistry in the same and different skin sources.

The *a*, *b* and luminosity scales on the chromameter detected changes in the skin response to all formulations of 0.05% betamethasone dipropionate with less variability, over all time intervals, than the visual skin blanching or the tape-stripping methods. The lowest variability among all techniques evaluating corticosteroid activity was observed 8 h after drug application

The data demonstrate that using the coefficient of variation as the basis for choosing a method for assessment of topical corticosteroid bioequivalence is inappropriate. Indeed, the low variability in the luminosity and *b* scales on the chromameter reflect the lack of sensitivity of these scales to discriminate the skin response to five formulations of topical 0.05% betamethasone dipropionate in the present study. This lack of discrimination power with the two scales is in contrast to the rank order of these products measured with the visual skin blanching assay in the present study and by Cornell and Stoughton (1984).

The moderate correlation between the tape-stripping method and the skin blanching response ($r = +0.6$) and the excellent correlation between the *a* scale on the chromameter and visual skin blanching ($r = -0.9$) demonstrate their potential usefulness in bioequivalence testing of topical corticosteroids. Application of these objective, sensitive, noninvasive and standardized methods to bioequivalence assessment of topical corticosteroids may further elucidate those interrelationships between vehicle formulation and chemical structure on drug delivery to the skin and the resulting pharmacological activity in vivo.

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