Comparison of Skin Stripping, *In Vitro* Release, and Skin Blanching Response Methods to Measure Dose Response and Similarity of Triamcinolone Acetonide Cream Strengths from Two Manufactured Sources

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ABSTRACT: The collective studies compare *in vitro* drug release, *in vivo* skin stripping, and skin blanching response methods for dose responsiveness and bioequivalence assessment of triamcinolone acetonide cream products, as a function of application duration, drug concentration, and manufacturer source. Commercially available triamcinolone acetonide creams (0.025%, 0.1%, and 0.5%) from two manufacturers were evaluated *in vitro* for rate and extent of drug release across synthetic membranes and *in vivo* for rate, extent, and variability of drug uptake into human stratum corneum and skin blanching response in human forearm skin. Data demonstrate that increasing triamcinolone acetonide cream concentration applied increased the rate and extent of drug released *in vitro* as well as the extent of drug uptake and skin blanching response in human skin *in vivo*. No difference ($p < 0.05$) between the two sources of 0.1% or 0.5% creams was measured by the skin stripping or skin blanching response methods. Dermatopharmacokinetic analysis of triamcinonide acetonide *in vivo* is therefore dose responsive to drug concentration applied and application duration and agrees with *in vivo* skin blanching results. Data support the use of dermatopharmacokinetic methods for bioequivalence and bioavailability assessment of topical drug products.
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INTRODUCTION

Topical glucocorticosteroids are the most widely used products in dermatology because of their favorable effects on inhibition of immune responses and cellular proliferation associated with cutaneous inflammatory diseases.[1,2] Dermatologic corticosteroids induce a skin blanching response associated with drug-induced constriction of cutaneous vessels that was first described by McKenzie and Stoughton[3] and used for the past 30 years to establish potency and bioequivalance of this drug class.[4] Increasing the drug concentration in a vehicle was assumed to increase drug delivery into the skin, thereby increasing the skin blanching response activity and hence, the therapeutic activity of the drug in human skin. Failure of some multiple strength products, such as 0.025%, 0.1%, and 0.5% triamcinolone acetonide

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creams, to demonstrate dose responsive behavior in previous human vasoconstrictor studies,[4] has led some investigators to conclude that there was no difference in drug released between the products, or no difference in the drug delivery to the skin between the products, and/or that the visual skin blanching response was a relatively insensitive measure of pharmacodynamic activity of the topical corticosteroids.[5,6]

Heretofore, probing the mechanistic basis of these issues has been difficult because of the lack of appropriate noninvasive methods by which drug released from the topical formulation and drug uptake into skin *in vivo* could be quantified with adequate analytical sensitivity and its validation against already established vasoconstrictor methods. The rate and extent of drug released from topical products can now be quantified *in vitro* using synthetic membranes.[7,8] Drug uptake into the stratum corneum *in vivo* can be measured by harvesting the stratum corneum previously exposed to a topical product with adhesive discs, which are subsequently extracted and quantified for drug concentration by a validated high-pressure liquid chromatography (HPLC) assay.[9–13] The latter method, also called skin stripping, has been described in previous bioavailability studies of topical betamethasone dipropionate[9–12] and hydrocortisone[13] from various commercial vehicle products in human ventral forearm stratum corneum. It is reproducible for stratum corneum harvesting,[9] sensitive to vehicle influences on drug delivery,[9,11] and correlates well with the resulting pharmacodynamic response in human skin *in vivo*.[9–11,13]

In the current studies, the dose responsiveness of three triamcinolone acetonide cream strengths from a single manufacturer was evaluated using *in vitro* drug release assay, *in vivo* dermatopharmacokinetics, and a modified *in vivo* skin blanching response assay and compared with previous work using the McKenzie–Stoughton vasoconstrictor assay.[4] In addition, the three methods were used to compare and contrast the bioavailability and bioequivalence assessment of two different manufacturers of the same drug strength in different vehicle formulations.

EXPERIMENTAL SECTION

Materials

The reference triamcinolone acetonide cream products (0.025%, 0.1%, and 0.5%; Bristol-Squibb) and test products (0.1% and 0.5% Fougera) were used as purchased from the University of Utah Health Sciences Center Pharmacy. The 0.025% test product was not available at the Health Sciences Center Pharmacy. Similar size tubes of the various products were purchased with expiration dates within 6 months of each other. The qualitative vehicle composition of the triamcinolone acetonide products within a manufacturer source was similar for all drug concentrations, but different between the two manufacturers (Table 1). For example, the reference products contained propylene glycol in all drug strengths, but the test products did not. Nonetheless, the labeled vehicle composition for all drug strengths within each manufactured source was the same.

The skin blanching response in human ventral forearm skin was objectively quantified using a Minolta Chroma Meter model CR200 (Minolta Light Division, Ramsey, NJ). Reflected color from the skin was measured in the L*a*b* uniform color spaces recommended by CIE (Commission Internationale de l'Eclairage) in 1976 as the method to more closely represent perceived color and color difference. In the current studies, the "a*" scale values are reported. This color scale has been previously demonstrated to correlate well with the skin blanching response across many corticosteroids in a variety of vehicle bases.[9–12,14]

Ventral forearm stratum corneum was harvested with adhesive discs either prepared in our laboratory or purchased commercially. An 8-mm-diameter disposable biopsy punch (Acu-Punch®; Acuderm, Ft. Lauderdale, FL) was used to generate multiple discs from Transpore™ adhesive tape (3M, St. Paul, MN) in our laboratory by trained staff. D-squame® adhesives discs were purchased as a unit of 10 1.3-cm-diameter D-squame® adhesive discs (CuDerm Corp., Dallas, TX) on a polymer-backing sheet. Both adhesive systems removed similar ($p < 0.05$; Wilcoxon signed rank test) amounts of stratum corneum per surface area of the adhesive disc ($\mu g\ cm^{-2}$) when evaluated in triplicate at side-by-side skin sites on the ventral forearms of three subjects (data not shown). Both adhesive systems also produced similar drug recoveries from five replicate extracted, triamcinolone acetonide-spiked adhesive disc calibration standards used in the HPLC assay (data not shown). Protective nonoccluding guards were prepared as described previously,[9] with either one or two inclusive skin test areas, as required by the study design.

Table 1. Vehicle Composition of Study Triamcinolone Acetonide Creams

Status	Drug Concentration	Vehicle Composition
Reference	0.025%	Propylene glycol
	0.1%	Cetearyl alcohol
	0.5%	Ceteareth-20
		White petrolatum
		Sorbitol solution
		Glyceryl monostearate
		Polyethylene glycol monostearate
		Simethicone
		Sorbic acid
		Purified water
Test	0.1%	Emulsifying wax
	0.5%	Cetyl alcohol
		Isopropyl palmitate
		Sorbitol solution
		Glycerin
		Lactic acid
		Benzyl alcohol
		Purified water

Triamcinolone acetonide (99.8% purity) used in the HPLC assay was used as purchased from Sigma (St. Louis, MO). HPLC-grade acetonitrile was used as purchased from Baxter Scientific, Salt Lake City, UT. Mixed cellulose acetate/nitrate synthetic membranes (HAWP), 25-mm diameter, 0.45-μm pore size, were purchased from Millipore Corp., Boston, MA. Phosphate buffered saline pH 7.4 was prepared in our laboratory and used in the receiving chamber reservoir of the *in vitro* release studies. Modified flat flange Franz cells with a diffusing surface area of 0.64 cm^2, and a total receiving chamber volume of 5 mL were purchased from Crown Glass, Somerville, NJ. Plexiglass rings were manufactured at the University of Utah and used to ensure the same dose (200 μL) to all *in vitro* cells. The rings were made with the specifications of an internal diameter of 1.6 cm, a height of 0.2 cm, a surface area of 2.01 cm^2, and a volume of 0.201 cm^3. The dose applied per diffusion surface area of the diffusion cell was therefore, ~313 μL product cm^{-2}.

Caution: *The following chemicals and biologics are considered hazardous and should be handled and disposed of carefully: acetonitrile, human skin strippings, respectively*.

Methods

In Vitro Drug Release

In vitro triamcinolone acetonide release profiles from 0.025%, 0.1%, and 0.5% reference creams as well as 0.1% and 0.5% test creams were evaluated over a 6-h time period. A nonoccluded dose of ~313 μL product cm^{-2} diffusing surface area applied in a Plexi glass ring fitted to the modified Franz cell diffusion chamber with diffusing surface areas of 0.64 cm^2. All diffusion chamber *in vitro* release experiments were performed at 32°C. Cellulose acetate/nitrate membranes were presoaked 0.5 h in the phosphate buffered saline (pH 7.4) before use in the release experiments. Two hundred microliter samples were collected from the receiving chamber before drug application (0 h) and 0.5, 1, 2, 4, and 6 h after drug application. Fresh 32°C phosphate buffered saline (200 μL) was used to replenish to the total receiving chamber volume in the diffusion chamber after each sample collection.

Drug concentrations from samples collected in the release experiments were calculated from the best-fitted linear regression (r^2) of a known aqueous standard curve (1–500 μg mL^{-1}) using the area-under-the-peak method (AUP) of the drug peak. Total actual amount of drug released (μg cm^{-2}) was calculated based on the surface area of diffusion (0.64 cm^2), total receiving chamber volume, and the dilution factor of the drug concentration in the receiving chamber over time, due to replenishment of the sample volume removed with new receiving chamber solution.[15] The amount of drug released (μg mL^{-1}) in the diffusion cell samples was determined using the peak area method of the drug peak from the HPLC analysis and the slope and y intercept of the linear regression of known aqueous triamcinolone acetonide standards. The dilution factor was determined from the receiving chamber total volume and the sample volume collected and replaced with fresh receiving chamber solution at each time point.

For each diffusion cell, drug release (μg cm^{-2}) as a function of square root of time was determined. The drug release profile was best fitted by least squares linear regression (r^2) using the time points over the square root time period of 0.5 to 6 h. The mean ± SD of the slope of triamcinolone acetonide release from three cells for each product was determined. The rate of drug released and extent of drug released in each of the cells was statistically evaluated for significance between

products by the unpaired nonparametric Mann-Whitney test (version 4.1, Statview™; Abacus Concepts, Calabassas, CA).

In Vivo Human Subject Studies

Research with human subjects followed the tenets of the Declaration of Helsinki Promulgated in 1964 and was approved by the University of Utah Institutional Review Board. Human subjects were enrolled in the associated studies after providing informed consent.

Five to 10 human subjects composed of male and female were enrolled in each of the studies described below, after providing informed consent. All studies were approved by the University of Utah Institutional Review Board. All enrolled subjects were healthy Caucasians, as determined by the investigator, 19–65 years of age, male or female. Exclusion criteria included smokers, strenuous exercise (running, aerobics, power walking, or bicycling), caffeine or alcohol consumption over the 24–48 h of the study, current skin disease, or use of topical drug medications. Enrolled subjects received remuneration for their participation in the respective studies.

A template was used to demarcate the test skin sites on the ventral forearm of each subject that ensured all skin sites were located within an area that was 3 cm above the wrist and 3 cm below the antecubital fossa in all human studies. Skin sites were spaced 2 cm center-to-center apart on the ventral forearm. Skin sites were demarcated by four dots around its circumference using an indelible marking pen.

Dose Response Study: Application Duration

Ten subjects, five males (35 ± 10 years, mean $\pm$ SD) and five females, (29 ± 11 years, mean $\pm$ SD) were enrolled and completed the study after providing informed consent. Five 1-cm-diameter circular skin sites were demarcated on the ventral forearm of each subject. The five skin sites were randomly assigned to an application duration of 0, 0.5, 1, 2, or 6 h.

A dose of 5 μL of the reference triamcinolone acetonide cream, 0.05%, was dispensed synchronously to four skin sites to produce a typical dermatological dose.[5,10] The product was dispensed via a preloaded 250 μL glass Hamilton syringe, which has been previously demonstrated to deliver 5.8 ± 0.4 mg of product (mean $\pm$ SD, $n = 10$). One skin site designated as "0 hour," representing the control site, was not treated. Drug treatment was applied synchronously and removed asynchronously. All designated skin sites were protected with a nonoccluding guard for the duration of the designated application duration.[9]

Dose Response Study: Product Strength and Manufacturer Source

Three females (31 ± 12 years, mean $\pm$ SD) and three males (25 ± 3 years, mean $\pm$ SD) were enrolled and completed the study. Because of the high temporal variability inherent to skin, the dose response and bioequivalence comparisons between chemically similar products were performed in the same subject, under the same experimental conditions, at the same time. Five circular skin sites, each 3.8 cm^2 surface area, were demarcated on each ventral forearm of each subject. Application of the 0.025%, 0.1%, and 0.5% reference triamcinolone acetonide creams and the 0.1% and 0.5% test creams to one of the five skin sites was randomized for each subject. The randomization pattern of the products on the left arm skin sites was identical to the right arm. The right arm was used for dermatopharmacokinetic evaluation of drug uptake using the skin stripping method for the harvesting of drug-treated stratum corneum and the left arm was used for the skin blanching response evaluation by a reflectance colorimeter a* scale in all subjects. Each skin site received a 10-μL dose of the assigned product over a skin surface area of 3.8 $cm,^2$ representing a typical dermatological corticosteroid dose[10] of ~3 mg product cm^{-2}. Drug treatment was applied synchronously and removed asynchronously. All drug-treated and untreated skin sites were protected with a nonoccluding guard.[9]

Intra-Subject Variability Study

Four females (aged 43 ± 5 years, mean $\pm$ SD) and one male (age 23 years) were enrolled and completed the study. Six circular skin sites, each 0.8 cm^2 surface area, were demarcated on the right ventral forearm. One site was randomly assigned as an untreated skin site. The other five skin sites received a 5-μL dose of the reference 0.5% triamcinolone acetonide cream over a skin surface area of 0.8 cm^2 for 2 h. Drug treatments were applied synchronously and removed asynchronously. Drug-treated and untreated skin sites were protected during product application with a protective, nonoccluding guard.[9]

Skin-Stripping Method

At the end of the assigned dose (application duration), the residual drug was removed from the drug-treated skin site with three individual dry cotton-tipped wood applicators. The stratum corneum at the treated and untreated control skin sites was removed for analysis of drug content using a skin-stripping procedure previously developed and validated to remove ~75% of the stratum corneum within each subject.[9,11] The first adhesive disc applied to a designated skin site was discarded because of potential residual drug contamination. The remaining nine adhesive discs collected from the designated skin site were combined in a capped 1.7-mL polypropylene conical microcentrifuge tube and stored at −70°C until analyzed for drug content by HPLC.

Skin Blanching Response Assessment

The pharmacodynamic activity of triamcinolone acetonide in human skin *in vivo* was measured using the surrogate marker, the skin blanching response, also known as the vasoconstriction response.[3] The skin blanching response was measured as the loss of skin color at the treatment site by the reflectance colorimeter a* scale.[9–12] Thus, increasing skin blanching responses are associated with decreasing colorimeter a* scale values. The skin blanching response was measured simultaneously in the above subjects at baseline, immediately after residual product removal, and again 2, 4, 6, 18, 20, 22, and 24 h after residual product removal, using the reflectance colorimeter a* scale. The same products were simultaneously evaluated for drug uptake into stratum corneum on the opposite forearm of each subject.

The designated skin sites on the left arm were evaluated with the colorimeter 1 h before product application (baseline), immediately after residual product removal of a 2-h treatment and again 2, 4, 6, 18, 20, 22, and 24 h after residual product removal. In this way, the skin blanching response(s) over a 24-h time period were assessed for each strength and manufacturer of triamcinolone acetonide cream evaluated. Reflectance colorimeter a* scale data from drug-treated skin sites were baseline-adjusted for inherent differences in skin color along the ventral forearm. Data are presented as the area-under-the baseline adjusted a*-time curve (AUEC 0–24 h a* scale) and were calculated using the trapezoidal rule.[16]

Analytical Method

Skin-Stripping Samples Extraction

The nine combined skin strippings from each skin site and known drug-spiked adhesive disc calibration standards were extracted with 0.75 mL of acetonitrile. Triamcinlone acetonide in the skin strippings and spiked calibration standards were extracted at high speed on a tabletop vortexer for 1 min, resting 10 min, then centrifuged at 8000 rpm for 10 min. The organic extract was then transferred to a clean, labeled 1.7-mL polypropylene microcentrifuge tube and dried under air in a fume hood. The dried extract was resuspended in 200 μL of HPLC mobile phase and 50 μL injected onto the HPLC via an autosampler (ThermoSeparations AS3000, San Jose, CA) . This extraction method produced recoveries of $> 90\%$ for a known extracted, triamcinolone acetonide-spiked adhesive disc calibration standard curve over the concentration range of 0.25 to 10.00 μg mL^{-1}. Recovery and linearity of the extracted triamcinolone acetonide-spiked adhesive disc calibration standards were not significantly different ($p < 0.05$) between the Transpore® or D-squame® brand adhesive discs (data not shown).

HPLC Assay

The HPLC system consisted of a binary HPLC pump (ThermoSeparations P2000, San Jose, CA) delivering a mobile phase composed of 40:60 (v/v) acetonitrile/water, at 0.7 mL min^{-1} flow rate through a 25°C heated, 4.6 mm × 12.5 cm C18 RP 5 μ particle column (Whatman, Clifton, NJ). Triamcinolone acetonide was detected at 254 nm with a forward optical scanning detector (Spectra Focus™ detector; ThermoSeparations) and quantified using the peak area (AUP) method. Retention time of triamcinolone acetonide in the assay was 5.5 min. Inter-run precision of the extracted, triamcinolone acetonide-spiked adhesive disc standard curve was $r^2 = 0.990 \pm 0.005$, mean ± SEM, $n = 4$) over a concentration range of 0.25 to 10.00 μg mL^{-1}. Limit of quantification of the extracted standards was 0.25 μg mL^{-1}. Intra-run precision of extracted, triamcinolone acetonide-spiked adhesive disc calibration standards ranged from 1.9% to 2.9%.

Drug concentrations in the unknown samples were determined against extracted, known triamcinolone acetonide-spiked, adhesive disc calibration standards submitted to the same extraction procedure as the unknown skin strippings.

Statistics

Data generated in the *in vitro* and *in vivo* drug concentration studies and the comparative studies of similar drug concentration between two manufacturers were analyzed statistically by the nonparametric Wilcoxon signed rank test and Mann-Whitney tests, respectively. The dose response studies were analyzed by analysis of variance (ANOVA) (Fishers PLSD) with Statview™ (version 4.1; Abacus Concepts) statistical computer software.

RESULTS

In Vitro Drug Release Profiles

In these preliminary studies, the mean *in vitro* triamcinolone acetonide release across a synthetic membrane increased with the square root of time ($h^{0.5}$) for all cream strengths (Table 2). Increasing triamcincolone acetonide concentration in the reference products evaluated from (0.025% to 0.1% and 0.5%) resulted in an increased rate ($\mu g\ cm^{-2}gh^{-0.5}$) and extent of drug released (total μgcm^{-2}) across the synthetic membrane. The rate and extent of drug released, however, was not consistently proportional to the increase in drug concentration applied. For example, increasing triamcinolone acetonide concentration applied 4-fold from the 0.025% to 0.1% reference creams produced a 3-fold increase in the rate of drug released and a 2-fold increase in the extent of drug released. Increasing the drug concentration in the reference creams 5-fold from 0.1% to 0.5% produced only a 1.5-fold increase in both the rate and extent of drug released.

Increasing the drug concentration of the test cream products 5-fold from 0.1% to 0.5% produced a 3-fold increase in the rate of drug released and a 4-fold increase in the extent of drug released. Thus, although increasing the drug concentration in these topical triamcinolone acetonide reference and test cream products resulted in an increased rate and extent of drug release *in vitro*, it was not always proportional to the increase in labeled strength of the products.

Table 2. Triamcinolone Acetonide Release *In Vitro* Across a Synthetic Membrane

Product (%)	Rate of Drug Release ($\mu g^* cm^{-2}*h^{0.5}$)[a]	Extent of Drug Release (Total $\mu g/cm^2$)[a]
Reference		
0.025	1.00 ± 0.10	3.33 ± 0.17
0.1	3.32 ± 0.99	6.57 ± 1.55
0.5	4.99 ± 0.62	9.70 ± 0.70
Test		
0.1	1.56 ± 0.24[b]	2.97 ± 0.38
0.5	4.42 ± 0.48	10.90 ± 1.82

[a] Mean ± SD of three cells.
[b] $p < 0.05$ Mann-Whitney test.

Comparison of the rate and extent of drug released across the synthetic membrane with two sources of 0.1% triamcinolone acetonide cream revealed 2-fold greater rate and extent of drug released with the reference cream that was statistically greater than the test cream ($p < 0.05$; Mann-Whitney test). In contrast, the rate and extent of drug release with the reference and test 0.5% creams was not significantly different. These preliminary results indicate that drug release comparisons across multiple strengths of topical drug products and between manufacturers can be different when the vehicle composition is not qualitatively and quantitatively the same and, therefore, cannot be assumed to be the same as a function of labeled strength.

Whether the differences and similarities between the two manufacturers of 0.1% and 0.5% triamcinolone acetonide creams *in vitro* would predict differences in the *in vivo* drug uptake or the skin blanching responses in the ventral forearm skin was evaluated in subsequent human studies.

In Vivo Human Studies

Dose Response: Application Duration

The dermatopharmacokinetic profile of drug uptake into human stratum corneum was evaluated with the reference 0.05% triamcinolone acetonide cream in order to optimize the time points of data collection. The highest cream strength was used in these studies to maximize the analytical detection and quantification of triamcinolone acetonide in the stratum corneum. Drug uptake as a function of application duration (hours) was investigated in 10 subjects, including five females and five males. Figure 1 illustrates the mean pooled (heavy solid line; $n = 10$), as well as the individual profiles (thin lines) of triamcinolone acetonide uptake into the stratum corneum as a function of product application duration. The profile of drug uptake was variable between subjects, but demonstrated a mean increased triamcinolone acetonide uptake with increased dose (application duration on the skin surface) up to 2 h. No further increase in mean drug uptake in human skin was

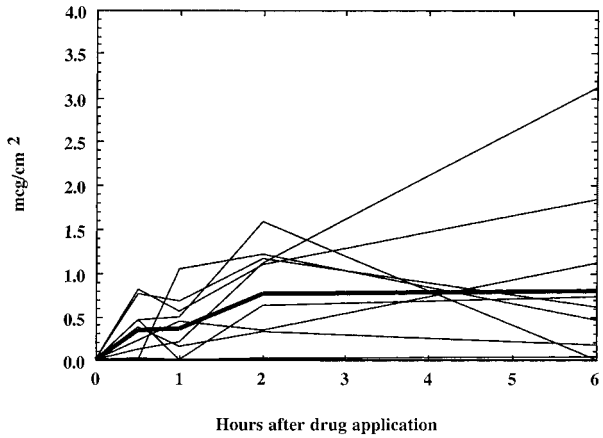

Figure 1. Uptake of triamcinolone acetonide into human stratum corneum *in vivo* from reference 0.5% cream product as a function of application time (hours). Drug uptake (μg) is normalized per square centimeter of adhesive disc surface area (μg/cm^2). Thin lines, individual subjects; heavy solid line, mean of 10 subjects.

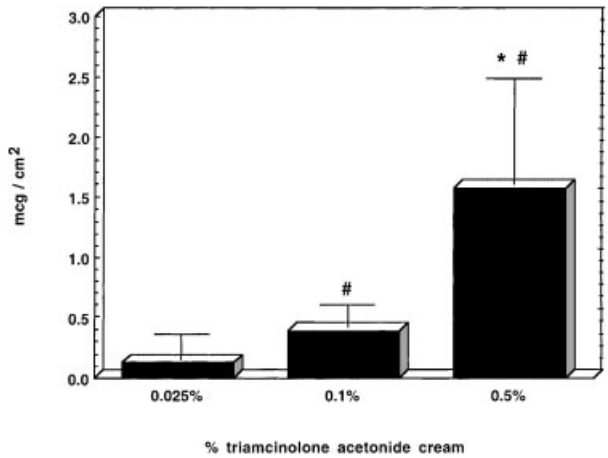

Figure 2. Dose response relationship of triamciolone acetonide cream concentration applied and the resulting drug content in human ventral forearm stratum corneum *in vivo* using the skin-stripping method. Mean ± SD, $n = 6$. *$p < 0.05$ from 0.1%, #$p < 0.05$ from 0.025%.

observed from 2 h to 6 h period of treatment ($p < 0.05$; ANOVA). These data support a "steady state" and maximal uptake of triamcinolone acetonide into stratum corneum within 2 h of product application reflecting the saturation of the stratum corneum with soluble drug from the applied product and the equilibrium between continued uptake and elimination of applied drug from this compartment over time. Drug uptake in females was not significantly different from males in this small population of 10 subjects ($p < 0.05$; ANOVA). Therefore, triamcinolone acetonide uptake into human stratum corneum *in vivo* is dose responsive when the product is left on the skin for increasing periods of time.

Dose Responsive: Product Strength

Increasing triamcinolone acetonide concentration of the reference cream product applied to human ventral forearm skin from 0.025% to 0.1% and 0.5% was associated with a dose proportional increase in mean drug uptake (Fig. 2). The stratum corneum triamcinolone acetonide content achieved with the 0.1% cream was statistically greater than the drug content produced with the 0.025% ($p < 0.05$) and the 0.5% cream was statistically greater than both the 0.1% and 0.025% creams ($p < 0.05$). Despite the between-subject variability in drug content with application of a single cream strength, each subject demonstrated a dose response profile to the multiple strengths of the triamcinolone acetonide creams applied (data not shown).

The pharmacodynamic activity of triamcinolone acetonide in human skin, as measured by the skin blanching response, was measured over a 24-h time period on the opposite forearm of the above subjects, using the a* scale of the L*a*b* uniform color space on the reflectance colorimeter. Figure 3 illustrates decreasing area-under-the-effect-time curve (AUEC 0–24 h) baseline-

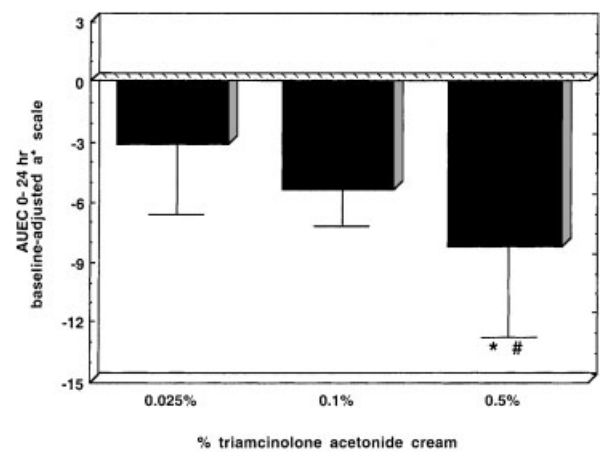

Figure 3. Dose response relationship of triamcinolone acetonide cream concentration applied and the resulting AUEC 0–24 h skin blanching response in human ventral forearm skin as measured by reflectance colorimetry a* scale. Mean ± SD, $n = 6$. *$p < 0.05$ from 0.1% cream, #$p < 0.05$ from 0.025% cream.

adjusted a* scale values, reflecting the loss of skin color (associated with an increased visual skin blanching response) with increasing triamcinolone acetonide concentration applied. Thus, the mean skin blanching response measured with reflectance colorimetry was dose responsive to increasing triamcinolone acetonide creams applied to human skin *in vivo*. The AUEC 0–24 h skin blanching response to the 0.5% cream was statistically greater ($p < 0.05$; ANOVA) than the 0.025% cream, but not the 0.1% cream. The AUEC 0–24 h skin blanching response to the 0.1% cream was not statistically different from 0.025% cream. Nonetheless, the mean AUEC 0–24 h values produced by the three strengths of triamcinolone acetonide cream produced dose responsive behavior that reflected the mean drug contents measured with the skin stripping method. Thus, increasing the triamcinolone acetonide cream concentration applied produced a dose dependent increase in drug uptake into the stratum corneum and skin blanching response *in vivo*.

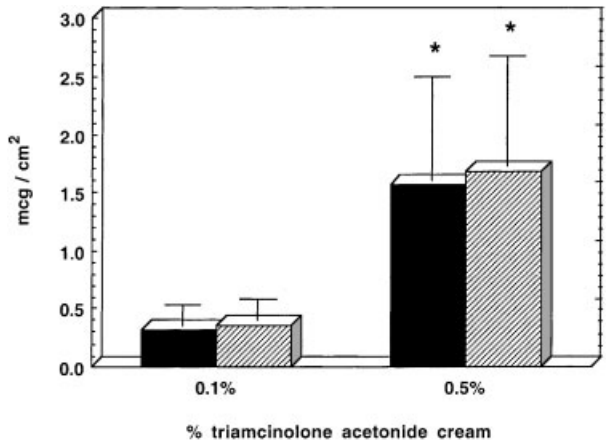

Figure 4. Triamcinolone acetonide content (μg/cm^2) in human stratum corneum *in vivo* after 2-h dose with test and reference triamcinolone acetonide cream products. Filled bar, reference product; striped bar, test product. Mean ± SD of $n = 6$, $^*p < 0.05$ from the 0.1% matched manufacturer source.

Manufacturer Source Comparison

Drug uptake with 0.1% and 0.5% triamcinolone acetonide creams from different manufacturer sources (reference and test) was evaluated simultaneously after 2 h of application on the ventral forearms of six human subjects. Triamcinolone acetonide uptake from the test and reference 0.1% triamcinolone acetonide creams was 0.48 ± 0.33 and 0.39 ± 0.33 μg/cm^2 (mean ± SD, respectively) and not significantly different ($p < 0.05$; Wilcoxon signed rank test) (Fig. 4).

Application of the test and reference products of 0.5% triamcinolone acetonide cream also produced similar drug contents ($p < 0.05$) in human stratum corneum (1.68 ± 0.94 vs. 1.56 ± 0.89 μg/cm^2, respectively) (Fig. 4). The mean ratio of test/reference drug uptake for the 0.1% and 0.5% triamcinolone acetonide creams was 1.23 and 1.08, respectively. Thus, application of the two cream products from different manufactured sources with different vehicle composition, yet the same drug strength, produced similar triamcinolone acetonide contents in human skin *in vivo*. Increasing the drug strength from 0.1% to 0.5% produced dose responsive increased stratum corneum triamcinolone acetonide concentrations with both manufactured sources. Both sources of 0.5% triamcinolone acetonide cream produced statistically greater triamcinolone acetonide contents in the harvested stratum corneum than their manufactured source-matched 0.1% cream sources ($p < 0.05$).

Skin Blanching Response

The skin blanching response produced from a 2-h dose of 0.1% and 0.5% triamcinolone acetonide creams from two different manufacturers (test and reference) was evaluated simultaneously on the ventral forearms of six human subjects. AUEC 0–24 h skin blanching responses, as measured by reflectance colorimetry a* scale values from the test and reference 0.1% triamcinolone acetonide creams were not significantly different (-5.28 ± 4.11 and -5.41 ± 2.06) (mean ± SD), respectively ($p < 0.05$; Wilcoxon signed rank test) (Fig. 5). Application of the test and reference products of 0.5% triamcinolone acetonide cream also produced similar AUEC 0–24 h skin blanching responses (-8.21 ± 3.20 and -8.20 ± 4.49, respectively) (Fig. 5). Both 0.5% triamcinolone acetonide creams produced statistically greater AUEC 0–24 h skin blanching responses than their matched 0.1% cream sources ($p < 0.05$).

The mean ratio of test/reference AUEC 0–24 h skin blanching responses for the 0.1% and 0.5% triamcinolone acetonide creams was 0.98 and 1.00, respectively. Thus, application of two triamcinolone acetonide cream products that differ in vehicle composition produced similar drug contents in the stratum corneum and similar skin blanching responses in skin *in vivo*.

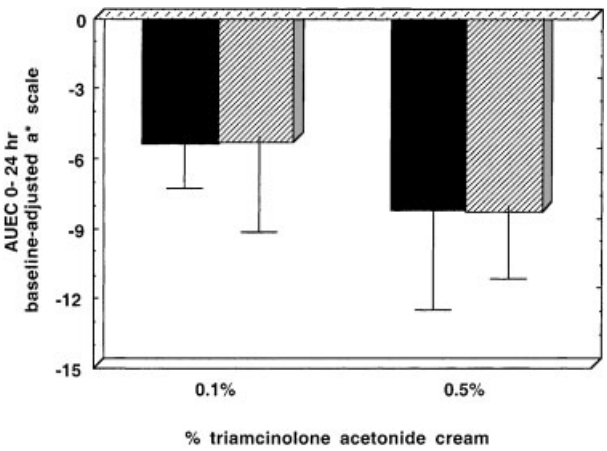

Figure 5. AUEC of the skin blanching response in human forearm skin *in vivo* after a 2-h dose of test and reference triamcinolone acetonide cream products. Solid bar, reference products; hatched bar, test products. Mean ± SD of $n = 6$ subjects.

The similarity of the test and reference 0.5% creams by the dermatopharmacokinetic and skin blanching response methods agrees with the similarity of the two sources by the *in vitro* drug release method. In contrast, whereas the test and reference 0.1% creams were similar with the skin stripping and skin blanching response *in vivo* methods, they were not by the *in vitro* drug release method. These data suggest that *in vivo* drug release methods do not always predict the drug uptake or drug activity of topical drug products in human skin *in vivo*.

Intra-Subject Variability

Variability in drug uptake and percutaneous absorption is widely known to be high between human skin sources.[9] The key to ascertaining the number of subjects required in a study to achieve statistical power to differentiate between products for a bioequivalence assessment, however, is the variability in the parameter measured within a subject, or intra-subject variability. Intra-subject variability will also influence the acceptance limits for the 90% confidence interval often used for assessing bioequivalence.[8,14,17] Therefore, an additional study was designed to measure the variability in drug uptake into the stratum corneum at five sites along the ventral forearm of the same individual using the reference 0.5% triamcinolone acetonide cream.

Variability in drug uptake data is a composite of the reproducibility in the stratum corneum removal by the skin-stripping method and the reproducibility in drug uptake within a subject along the ventral forearm. Previous validation in the amount of stratum corneum removed from three sites on the ventral forearm of three subjects averaged 26% when the adhesive discs used for harvesting the stratum corneum were weighed before and after skin stripping on an ultramicrogram (0.1 μg sensitivity) balance (data not shown). Analysis of the variability in drug uptake of a single dose of 0.5% triamcinolone acetonide cream at five sites ascending on the ventral forearm from the wrist to the antecubital fossa in five subjects demonstrates greater than 25% variability in drug content. The coefficient of variation in triamcinolone acetonide uptake is subject dependent, ranging from 21% to 67%, with a mean pooled variability of 38% (Fig. 6). Differences in the extent of drug uptake after a nonoccluded application for 2 h were not anatomical site dependent ($p < 0.05$; ANOVA), i.e., there was no association between extent of drug uptake and anatomical location along the forearm. The high intra-subject variability of triamcinolone acetonide uptake suggests that 32–40 subjects should be evaluated in a dermatopharmacokinetic study to achieve sufficient power (80%) to establish statistical significance between the two products based on a 90% confidence interval of 80–125% and a ratio of the mean test/mean reference ratio of 1.00.[18]

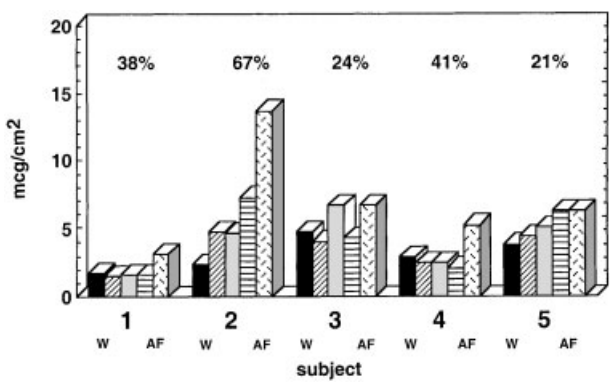

Figure 6. Intra-subject variability in triamcinolone acetonide uptake at five positions on the ventral forearm after a single 1-h dose of 0.5% cream. Filled bar, arm position #1; diagonal striped bar, arm position #2; stippled bar, arm position #3; horizontal striped bar, arm position #4; hatched bar, arm position #5. W, wrist; AF, antecubital fossa. The coefficient of variation (%) for each subject is printed above the respective data.

DISCUSSION

Failure of some multiple strength dermatologic corticosteroid products to demonstrate dose responsive behavior in the McKenzie-Stoughton vasoconstrictor assay[3,4] has led investigators to conclude that there was either no difference in drug released between the products, or no difference in the drug uptake into the skin between the products, and/or that the visual skin blanching response can be saturated and is a relatively insensitive measure of dermatologic corticosteroid pharmacodynamic activity of topical corticosteroids.[4–6]

Heretofore, probing the mechanistic basis of these issues has been difficult because of the lack of appropriate and noninvasive methods by which drug released from the topical formulation or drug uptake into skin *in vivo* could be quantified with adequate analytical sensitivity and validation against already established methods.[14] Methods are now available, however, to quantify the rate and extent of drug released from topical products *in vitro* using synthetic membranes[7,15] and drug uptake into the stratum corneum *in vivo* using a skin-stripping method, also called the dermatopharmacokinetic method.

Dermatopharmacokinetic data in the current study demonstrate that triamcinolone acetonide uptake into human stratum corneum *in vivo* is a function of treatment duration, drug concentration applied, and source of the manufactured products. Pseudo-steady-state triamcinolone acetonide content in the stratum corneum was achieved within 2 h of topical application of the cream products. The 2-h time required to achieve steady-state stratum corneum drug concentrations is longer than that previously observed with other compounds[19] reflecting differences in the kinetics of drug delivery in human versus rodent skin and suspension versus solution products. Increasing the concentration of triamcinolone acetonide cream applied was associated with a dose proportional increase in drug uptake into human stratum corneum, AUEC skin blanching response *in vivo*, and *in vitro* drug release. Thus, the *in vitro* drug release methods and *in vivo* pharmacokinetic and pharmacodynamic methods are dose responsive to various concentrations of the same drug within a manufactured source.

The discrepancy between the current dose proportional evaluation of three strengths of reference triamcinolone acetonide cream and the previous vasoconstrictor study,[4] likely reflects differences in the dose, application duration, assessment method of the skin blanching (vasoconstrictor) response, and the number of assessments made of the pharmacodynamic response over time. Previous studies with these products used the McKenzie-Stoughton vasoconstrictor assay study design, in which a single dose (~50 mg product per skin site) was applied for 16 h, removed, and visually assessed for the extent of vasoconstriction 2 h later. In contrast, the current study used a typical nonoccluded dermatological dose (~2–10 mg product cm^{-2} skin surface area) and the skin blanching response was measured objectively with a noninvasive reflectance colorimeter at multiple time points over a 24-h time period. Objective measurement of the skin blanching response at multiple time points over 24 h enables the inherent circadian activity of dermatologic corticosteroid activity in skin *in vivo*[6,10,12] to be incorporated into the overall assessment of pharmacodynamic activity, thereby improving the ability to discriminate between strengths of triamcinolone acetonide cream.

Although the three methods evaluated in the current studies demonstrated dose responsive behavior of the three strengths of triamcinolone acetonide creams from a single manufacturer, they did not agree in their assessment of similarity between manufactured sources of the 0.1% and 0.5% creams. No difference ($p < 0.05$) between two manufactured sources of 0.1% cream or 0.5% cream was measured *in vivo* as a function of the drug uptake or skin blanching response methods. The *in vitro* drug release method, however, demonstrated that whereas the two sources of 0.5% cream were similar, the two 0.1% creams were not. The lack of congruence in similarity assessment of the two 0.1% triamcinolone creams between the *in vivo* and *in vitro* methods may reflect differences in the vehicle compositions as well as the amount of soluble drug readily available for diffusion in the lower product strengths that is not an issue at the higher strengths. These products are suspensions and as such have both soluble and crystalline forms of the drug in the product(s). The relative ratio of soluble/crystalline form of the drug is influenced by the vehicle composition and drug concentration in the product. Although vehicle composition is similar among the drug strengths within a manufactured source, it is quite different between manufactured sources. Thus, the amount of soluble drug and thus thermodynamic activity in the different vehicle compositions must differ dramatically

between the products at low concentration, where a higher amount of drug is soluble, but not the higher concentrations, where the majority of the drug is crystalline. The amount of soluble drug in the two products thereby influences the rate and extent of drug released in the lower-strength products but not higher-strength products in the *in vitro* method. It is not surprising that drug release *in vitro* produces different kinetic profiles than drug uptake into stratum corneum *in vivo*. The *in vitro* method uses a synthetic membrane that is only semi-restrictive rate limiting, whereas the *in vivo* method uses a highly restrictive rate-limiting membrane, stratum corneum, which has unique lipid composition critical to barrier function that is not achieved with the synthetic membrane. Hence, the rate and extent of drug released *in vitro* reflects the drug diffusion through the vehicle and thus drug concentration gradients through the vehicle, whereas *in vivo*, it reflects drug partitioning into and drug concentrations through the stratum corneum.

Differences in bioequivalence assessment of two products between the *in vitro* and *in vivo* methods may also reflect the respective intra-membrane or intra-subject variability. The quantification of drug uptake into the stratum corneum *in vivo* as well as the skin blanching response are considered highly variable ($>25\%$ coefficient of variation) in the subject population evaluated. Indeed, the high intra-subject variability of stratum corneum triamcinolone acetonide concentrations in the current human dermatopharmacokinetic studies ($>25\%$ coefficient of variation) supports the evaluation of 32–40 subjects in a bioequivalence assessment study with products of similar drug concentration.[18] Bioequivalence assessment of dermatologic corticosteroids using the skin blanching response E_{max} model requires 40–60 subjects who demonstrate dose responsive behavior.[14] Thus, although the dermatopharmacokinetic and skin blanching response *in vivo* methods agree in terms of bioequivalence assessment of the two sources of 0.1% and 0.5% triamcinolone acetonide cream in the current small human population, a larger study would be required to achieve statistical power to confirm the assessment of bioequivalence.

In summary, data illustrate that a topical corticosteroid is dose responsive to application duration and concentration applied *in vivo* using a dermatopharmacokinetic method in which drug content in stratum is quantified. Dermatopharmacokinetic data demonstrate less variability between human subjects than the skin blanching pharmacodynamic response and is superior to *in vitro* drug release methods in differentiating between different strengths of triamcinolone acetonide. Further, bioequivalence between similar strength dermatologic corticosteroids using the *in vivo* dermatopharmacokinetic method agrees well with the *in vivo* skin blanching response method. Thus, these collective data suggest that the dermatopharmacokinetic method will be useful in probing bioavailability issues of dermatological products as a function of drug product strength and vehicle composition, and in the assessment of bioequivalence of similar drug products.

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