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RESEARCH PAPER

Cutaneous Pharmacokinetics of Acyclovir Cream 5% Products: Evaluating Bioequivalence with an *In Vitro* Permeation Test and an Adaptation of Scaled Average Bioequivalence

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ABSTRACT

Purpose The *in vitro* permeation test (IVPT) with a new statistical approach was investigated to evaluate the utility of an IVPT methodology as a sensitive tool to support a demonstration of bioequivalence (BE) for topical dermatological drug products.

Methods IVPT experiments were performed utilizing *ex vivo* human skin. The initial screening tests involved four differently formulated acyclovir 5% creams: the U.S. Zovirax® as the reference product and the U.K. Zovirax®, Aciclovir 1A Pharma® and Aciclostad® as test products. Subsequently, a pivotal BE study was conducted comparing the two Zovirax® creams. The resulting data was used to evaluate BE of test (T) versus reference (R), T versus T, and R versus R, with an adaption of scaled average BE approach to address high variability in IVPT data.

Results More acyclovir permeated into and through the skin from the two Zovirax® creams compared to the two non-Zovirax® creams. The U.S. Zovirax® cream showed a significantly higher J_{max} and total amount permeated over 48 h, compared to the U.K. Zovirax® cream. The statistical analysis indicated that the test and reference products were not bioequivalent, whereas each product tested against itself was shown to be bioequivalent.

Conclusions The current study demonstrated that the IVPT method, with an appropriate statistical analysis of the results, is a sensitive and discriminating test that can detect differences in the rate and extent of acyclovir bioavailability in the skin from differently formulated cream products.

KEY WORDS acyclovir · bioequivalence (BE) · *in vitro* permeation test (IVPT) · scaled average bioequivalence (SABE) · topical dermatological products

ABBREVIATIONS

ABE	Average bioequivalence
AUC	Area under the curve
BA	Bioavailability
BE	Bioequivalence
C_{max}	Maximum concentration
dOFM	Dermal open flow microperfusion
HPLC	High performance liquid chromatography
IVPT	*In vitro* permeation test
IVRT	*In vitro* release test
J_{max}	Maximum flux
LLOQ	Lower limit of quantification
PD	Pharmacodynamic
PG	Propylene glycol
PK	Pharmacokinetic
Q1	Qualitatively
Q2	Quantitatively
Q3	Physically and structurally
QC	Quality control
R	Reference
RLD	Reference Listed Drug

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SABE Scaled average bioequivalence
SC Stratum corneum
T Test
TEWL Transepidermal water loss

INTRODUCTION

The widespread availability of generic versions of many pharmaceutical drug products in the United States (U.S.) has had a profound economic and social impact. Based upon a recent report on generic drug usage in the U.S., 90% of the prescriptions filled in 2018 were dispensed as generics, amounting to 4 billion generic prescriptions dispensed and $293 billion in savings during 2018, and collectively representing nearly $2 trillion saved in healthcare costs over the preceding decade as a result of the availability of generic drug products (1). The magnitude of these savings is extraordinary on a national level, but is even more socially significant in the impact it has for individual patients. The cost of some brand name medications may be prohibitive for many individuals, including some of the most vulnerable patients in the population, like elderly patients on limited, fixed incomes. By contrast, the affordability of generic drug products makes them more accessible to patients (2), which can improve patient compliance with medication regimes and mitigate the individual, social and economic impact of the costly clinical interventions that might otherwise become necessary for conditions that remain untreated with medications.

The affordability of generic drug products is a direct consequence of highly efficient scientific and regulatory approaches utilized to develop most generic drug products, which typically rely upon a comparative pharmacokinetic (PK) study to evaluate whether the rate and extent to which the drug becomes available at or near the site(s) of action is the same for the generic product and the reference listed drug (RLD) product (3). This approach to evaluating comparative bioavailability (BA) is considered to be among the most accurate, sensitive and reproducible ways to demonstrate bioequivalence (BE) for a generic drug product.

For drugs that are intended to be delivered to the systemic circulation, as is the case for many oral dosage forms, this approach involves the PK sampling of the blood at multiple time points following dose administration, and measuring the concentration of the drug (or a related analyte) in the plasma or serum, which is a relatively straightforward process. However, for locally acting drugs, like those in topical dermatological drug products, evaluating the concentration of the drug at or near the site of action in the skin has been more challenging (4). As a result, the development of generic versions of topical drug products has typically instead relied upon comparative clinical endpoint BE studies, which are less accurate, sensitive and reproducible than PK studies and considerably more costly and time consuming. The expensive and inefficient development pathways to which most generic topical drug products have been relegated has represented a barrier to entry for generic drug developers and negatively impacted their availability and their affordability. The notable exceptions among topical dermatological drug products have been those that contain corticosteroid (glucocorticoid) drugs, for which a more efficient *in vivo* pharmacodynamic (PD) vasoconstrictor study has been recommended by the U.S. Food and Drug Administration for the demonstration of BE (5). As a result, although these corticosteroid creams, ointments and other topical dosage forms represent a small proportion of topical dermatological drug products overall, these RLD products often have multiple generic versions available. The hypothesis of this work was that if similarly efficient cutaneous PK BE methodologies could be developed, these methods could facilitate the greater availability of affordable generic topical dermatological drug products.

The feasibility of existing *in vitro* and *in vivo* cutaneous PK methodologies have been considered during recent decades, with each methodology offering different strengths and limitations (reviewed in Raney *et al.* 2015) (6). One of the most promising among these cutaneous PK methodologies is the *in vitro* permeation test (IVPT) methodology which has been shown to correlate well with *in vivo* results, particularly when the IVPT and *in vivo* study designs are harmonized (7,8). Notably, IVPT studies have specifically shown promise to correlate with and be predictive of *in vivo* assessments of BE (reviewed in Lehman *et al.* 2011) (9). The goal of this work was to evaluate whether an IVPT method could be utilized to compare the BA of acyclovir from different creams and support an evaluation of BE. *In vitro* PK endpoints related to the rate (i.e., the flux) and extent (i.e., the cumulative amount) of acyclovir permeation through the skin were identified. The maximum flux (J_{max}) at the peak of the acyclovir flux profile and the cumulative total permeation of acyclovir across the study duration, which corresponds to the area under the curve (AUC) of the incremental acyclovir permeation profile, were evaluated as cutaneous PK endpoints.

Pharmaceutically equivalent acyclovir cream, 5% products from different parts of the world were selected for the study (Table I). Acyclovir cream, 5% is a product with modest efficacy, for which the feasibility of a comparative clinical endpoint study is questionable, and for which no generic versions had been approved in the U.S., despite the fact that there were no unexpired patents or exclusivities (10). This model drug product was, therefore, representative of the many topical dermatological drug products for which the historical absence of an efficient generic drug product development pathway may have been the primary reason that affordable, generic versions were not available to patients. The comparison of a product to itself was intended to serve as a positive control for BE. By contrast, the comparison of non-U.S. acyclovir cream, 5% products with different formulation compositions and/or packaging configurations compared to the U.S. RLD were included in the study to evaluate the sensitivity of the IVPT methodology to discriminate potential differences in BA and/or BE for similar, pharmaceutically equivalent products.

Table I Comparison of Inactive Ingredients in the Screened Acyclovir Cream Products

U.S. Zovirax®	U.K. Zovirax®	1A Pharma®	Aciclostad®
Cetostearyl alcohol	Cetostearyl alcohol	Cetyl alcohol	Cetyl alcohol
Propylene glycol	Propylene glycol	Propylene glycol	Propylene glycol
Poloxamer 407	Poloxamer 407		
Sodium lauryl sulfate	Sodium lauryl sulfate		
Mineral oil	Liquid paraffin	Liquid paraffin	Liquid paraffin
White petrolatum	White soft paraffin	White Vaseline	White Vaseline
Water	Purified water	Purified water	Purified water
	Dimethicone	Dimethicone	Dimethicone
	Arlacel 165 (glycerol monostearate, macrogol stearate 100)	Polyoxyethylene stearate Glycerol monostearate	Macrogol stearate

A variety of statistical analyses were used to evaluate the IVPT results. Since the permeation of compounds through human skin is known to be highly variable, a replicate IVPT study design was utilized and the results were analyzed statistically using a novel adaptation of an approach previously developed to evaluate scaled average BE (SABE) for highly variable drugs. The IVPT BE statistical approach was developed to capitalize upon the ability of an IVPT methodology to sensitively discriminate differences in cutaneous BA through the skin from any single individual, while compensating for the variability from one individual compared to another in acyclovir permeation through the skin. The intention was to optimize the efficiency with which an IVPT study could evaluate BE by increasing the power of the comparative cutaneous PK study and reducing the number of individuals whose skin would be required, thereby reducing the size and cost for the IVPT study. The overall goal was to develop a more efficient, cutaneous PK methodology that could support the evaluation of BE for topical dermatological drug products.

MATERIALS AND METHODS

Materials

Sodium chloride, potassium phosphate, methanol, sodium borate salt, phosphoric acid and gentamicin sulfate were purchased from Fisher Scientific Inc. (Fair Lawn, NJ). Acyclovir reference standard (acycloguanosine) was purchased from Sigma Aldrich (St. Louis, MO). All reagents were of analytical grade or better. Nanopure water was supplied in-house by a Milli-Q® system (EMD Millipore; Billerica, MA).

The U.S. acyclovir cream, 5% RLD (Zovirax® in a tube; NDC 0187–0994-45) was purchased from Cardinal Health (Dublin, OH). Acyclovir cream, 5% products acquired from international research colleagues included a Zovirax® cream sold in the United Kingdom (U.K.) in a pump, as well as two Austrian acyclovir cream, 5% products in tubes (Aciclovir 1A Pharma® and Aciclostad®). The four acyclovir cream, 5% products each have a different inactive ingredient composition relative to the others, although the two Austrian creams are compositionally similar to each other (Table I).

Skin Preparation

All human abdominoplasty surgical waste skin pieces used for IVPT experiments were obtained from the Cooperative Human Tissue Network (CHTN). The fresh skin samples were dermatomed to a thickness of 260 ± 40 μm, removing subcutaneous fat and keeping the outer layers of skin containing stratum corneum (SC), viable epidermis and some dermis. The dermatomed skin was stored at −20°C. On the day of the experiment, skin was cut into a 4.84 cm^2 square to fit onto the diffusion cell and thawed for at least 30 min prior to use. The barrier integrity of each skin piece was tested by measuring transepidermal water loss (TEWL) using a cyberDERM RG-1 open chamber evaporimeter (cyberDERM, Inc.; Broomall, PA) prior to the experiment. Any skin piece with obvious signs of physical damage, stretch marks or a TEWL reading higher than 15.0 $g/m^2/h$ was excluded from the experiment.

In Vitro Permeation Test (IVPT)

A PermeGear flow-through In-Line diffusion system (PermeGear, Inc.; Hellertown, PA) with an automated fraction collector was used for IVPT experiments. Diffusion cells with a permeation area of 0.95 cm^2, and membrane supports to prevent the skin from sinking into the receptor chamber were utilized. The receiver solution was isotonic potassium phosphate buffer (pH 7.4) with 0.005% gentamicin. The flow rate of the receptor solution was approximately 0.22 mL/h (pump setting at 0.5 rpm). The dermatomed 4.84 cm^2 piece of skin was mounted in the diffusion cell with epidermis facing the donor compartment. Once the TEWL measurement for each skin piece was taken to ensure the skin integrity, a single dose of 15 mg/cm^2 of acyclovir formulation (0.75 mg of

acyclovir per cm^2) was applied using a positive displacement pipette. While the clinical dose is an important factor to consider when selecting the dose for IVPT, additional factors should be considered. For example, since the therapeutic dose for most topical products is very small amount, it can be difficult to apply such small amount and cover the entire permeation area in a consistent manner without high variability. In this study, the IVPT dose was selected by testing various dose settings of positive displacement pipette and simulating the dose application on skin. Although the selected dose of 15 mg/cm^2 might be slightly higher than the clinical dose of acyclovir, it was determined to be the minimum amount to cover the permeation amount in a consistent manner. After the cream was dispensed on top of the skin surface, the exposed polytetrafluoroethylene tip of the positive displacement pipette was used to gently spread the formulation over the entire permeation area of the skin. The dose application area was left open to the air without any occlusion for the entire duration of the experiment. The IVPT experiments were performed for 48 h with continuous sampling every 4 h. The duration of IVPT was chosen to adequately characterize the cutaneous PK of acyclovir, including J_{max} and a decline of permeation rate. The two parameters, J_{max} and the cumulative total permeation corresponding to the AUC of the acyclovir permeation profile, were chosen as two key parameters for BE assessment. They are analogous to the maximum plasma concentration (C_{max}) and the area under the curve of the concentration-time profile, respectively, which are the PK parameters used in BE assessment of drugs measured in systemic circulation (11). The resulting receiver solution samples were analyzed by high-performance liquid chromatography (HPLC). Among the four acyclovir cream, 5% products (Table I) that were initially screened using skin from two donors, the Zovirax® cream, 5% marketed in the U.K. (in a pump) was selected for expanded comparison with the U.S. RLD Zovirax® cream, 5% in an additional four donors, collectively representing a pivotal study using *ex vivo* human skin obtained from a total of six donors.

Extraction of Acyclovir from Skin

At the end of IVPT experiments, each skin section used for the experiment was removed from the diffusion cell and analyzed to determine the amount of acyclovir retained in the skin. First, the residual formulation on top of the skin surface was cleaned three times using a 70% isopropyl alcohol pad. Then, the permeation area of the skin (0.95 cm^2) was cut into small pieces and added to a conical tube. The extraction solvent, 3 mL of 0.05 M borate buffer (pH 9.1), was added to the tube. The tube was capped, covered with Parafilm® and sonicated for 10 min. After sonication, the tubes were placed on a shaker at 200 rpm for 24 h. The samples were then centrifuged at 20,800 x g and an aliquot of supernatant was used for HPLC analysis.

HPLC Analysis of Samples

The IVPT samples and skin extraction samples were analyzed on an HPLC system consisting of a Waters® Alliance e2695 separations module, a 2489 dual-wavelength absorbance detector, and Empower™ software (Milford, MA). A validated HPLC method was used to quantify the IVPT and extraction samples. A Waters® Symmetry™ C18 (5 μm, 4.6 × 250 mm) column with a Waters Symmetry™ C18 Sentry guard cartridge (5 μm, 3.9 × 2.0 mm) was used to elute acyclovir. The mobile phase used was either 5:95 (*v*/v) or 15:85 (v/v) methanol:50 mM sodium phosphate, pH adjusted to 6.0 with phosphoric acid. The flow rate was 1.0 mL/min. The acyclovir peaks were detected at 254 nm. The IVPT samples were centrifuged for 10 min at 20,800 x g, with or without dilution with methanol (9:1, *v*/v). The resulting supernatant was analyzed with a set of standards prepared in isotonic potassium phosphate buffer (pH 7.4) with 0.005% gentamicin:methanol (9:1, v/v) or in isotonic potassium phosphate buffer (pH 7.4), depending on the IVPT sample preparation procedure for HPLC analysis. The skin extraction samples were diluted 10x with water and were analyzed with a set of standards prepared in water. All samples and standards were injected in duplicate. The injection volume for IVPT samples and skin extraction samples along with their respective standards were 100 μL and 50 μL, respectively. The lower limit of quantification (LLOQ) was 0.005 μg/mL for IVPT diffusion samples and 0.01 μg/mL for tissue extraction samples, with a linearity range up to 10 μg/mL. The method (for both of the mobile phase compositions described above) was precise and accurate with intra- and inter-day variation less than 5% and accuracy between 97 to 104% for quality control (QC) samples and the LLOQ calibration standard.

Statistical Analysis

Statistical analyses, except for BE assessments, were performed using GraphPad Prism® software (GraphPad Software, Inc.; La Jolla, CA). A Student's t test was used for comparisons of J_{max} and the total amount permeated. The homogeneity of variance within a donor was evaluated using the Brown-Forsythe test (12). Differences were considered to be statistically significant when $p \leq 0.05$ and significant differences were indicated as follows: $*p \leq 0.05$; $**p \leq 0.01$; *** $p \leq 0.001$.

The evaluation of BE was based upon the natural log-transformed total amount penetrated (total AUC) and maximum flux rate (J_{max}). Since not all donors had the same number of available replicate skin sections, to establish a balanced data set, we utilized the maximum number of replicates (r = 4)

that were consistently evaluable across all treatments in all donors. For those donors having more than four replicates, four replicate values were randomly selected. For the sample of $n = 6$ donors, each with a minimum of r = 4 replicates each coming from the two acyclovir cream, 5% products evaluated in the pivotal study (U.S. Zovirax® cream in a tube as the reference product and U.K. Zovirax® cream in a pump as the test product), the donor-averages were calculated as:

$$I_j = \frac{1}{4}\sum_{i=1}^{4}\left(T_{ij} - R_{ij}\right), \quad j = 1, 2, \ldots, 6$$

where T_{ij}, R_{ij} are the observations from the i[th] replicate and the j[th] donor from the test and reference formulations, respectively. Averaging across the donor averages, gives the point estimate (geometric mean ratio, GMR):

$$\overline{I} = \frac{1}{6}\sum_{j=1}^{6} I_j.$$

The within-reference standard deviation (S_{WR}) was evaluated from the data as:

$$S_{WR} = \sqrt{\frac{\sum_{j=1}^{6}\sum_{i=1}^{4}\left(R_{ij} - \overline{R_j}\right)^2}{(r-1)n}}$$

(where $\overline{R_j}$ is the mean of the j[th] donor for the reference formulation) and was used as a cutoff point in the following way:

a For $S_{WR} \leq 0.294$, the test and reference formulations are declared bioequivalent if the (1-α) *100% 2 one-sided confidence interval $\overline{I} \pm t_{(n-1),\alpha/2} * \sqrt{\frac{S_I^2}{n}}$ (13) is contained within the limits $\left[\frac{1}{m}, m\right]$.

b. In the case that $S_{WR} > 0.294$, a scaled criterion is used. This is a similar to the FDA approach for the analysis of highly variable drugs (14) modified for the particular design. The hypotheses to be tested are:

$$H_0 : \frac{(\mu_T - \mu_R)^2}{\sigma_{WR}^2} > \theta$$

$$H_a : \frac{(\mu_T - \mu_R)^2}{\sigma_{WR}^2} \leq \theta$$

Where μ_T and μ_R are the population means of the test and reference formulations respectively, $\theta = \frac{(\ln(m))^2}{(0.25)^2}$ and m represents the choice of the bioequivalence limit. The two products are declared BE if the upper bound of the confidence interval for the quantity $(\mu_T - \mu_R)^2 - \theta\,\sigma_{WR}^2$ is less than or equal to zero. This criterion includes an additional constraint that the point estimate has to fall with the limits $\left[\frac{1}{m}, m\right]$. Rejection of the null hypothesis supports BE of the test and reference products.

RESULTS

In Vitro Permeation Test (IVPT)

When the four acyclovir creams were initially screened and compared by conducting IVPT experiments using skin obtained from two donors, the permeation of acyclovir into and through the skin from the two Zovirax® creams was much higher compared to the two non-Zovirax® acyclovir creams (Fig. 1). The two non-Zovirax® creams, 1A Pharma® and Aciclostad®, showed comparable permeation levels to each other. Many of the IVPT samples from the two non-Zovirax® acyclovir creams contained acyclovir below the LLOQ (0.005 μg/mL). Subsequent IVPT experiments were conducted to evaluate the cutaneous PK (i.e., permeation profiles) of only the two Zovirax® creams (Fig. 2). When the two Zovirax® creams were compared per each donor, the mean J_{max} value was higher for the U.S. Zovirax® cream for all donors, except for Donor 6 (Fig. 3). A significant difference ($p < 0.05$) in J_{max} between the two creams was observed in Donor 3 and Donor 4 (Fig. 3). The mean J_{max} and the total amount of acyclovir permeated over 48 h from six donors were both significantly ($p < 0.05$) higher for the U.S. Zovirax®, compared to the U.K. Zovirax® (Fig. 4a, b). The mean amount of acyclovir retained in the skin after the 48 h IVPT experiment was not significantly different between the two Zovirax® creams (Fig. 4c). The flux profiles from the two Zovirax® creams in individual skin sections from each of six donors, with four to seven replicates per donor are shown in Fig. 5. For both Zovirax® creams, the greatest intra-donor variability was observed from skin sections obtained from Donor 6, with p value of 0.0906 and 0.0120 for the U.S. and the U.K. Zovirax® creams, respectively (Table II). The inter-donor variability was not significant ($p > 0.05$) for the U.S. Zovirax® cream but was significant ($p = 0.0024$) for the U.K. Zovirax® cream (Brown-Forsythe test).

Statistical Analysis

Using the statistical analysis of BE and the resulting balanced data set of $n = 6$ donors and r = 4 replicates described above, three comparisons were performed; the test product versus the

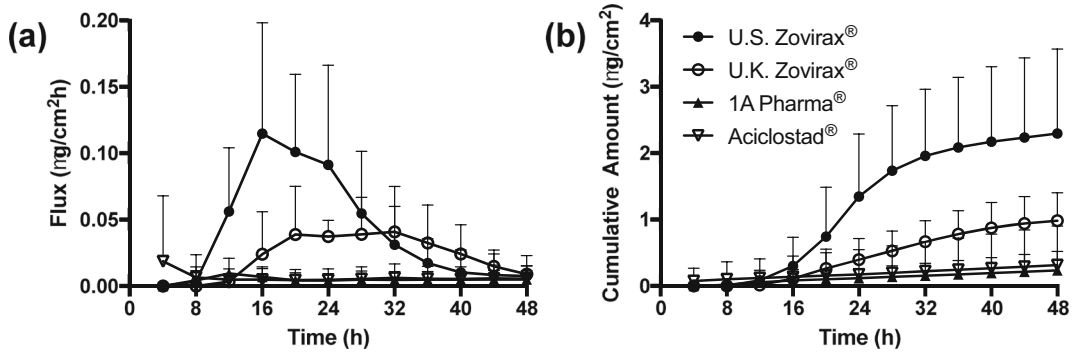

Fig. 1 (**a**) Flux profiles and (**b**) cumulative permeation levels of U.S. Zovirax®, U.K. Zovirax®, 1A Pharma®, and Aciclostad® acyclovir creams (Mean ± SD; n = 7–8 from two donors).

reference product (T vs R), the test product versus itself (T vs T) and the reference product versus itself (R vs R). The results are summarized in Table III. In all three comparisons, S_{WR} > 0.294 indicating that it was appropriate to use the scaled approach. The test and reference products were not BE, whereas both comparisons of the test versus itself and the reference versus itself yielded BE results.

The current data set was employed to evaluate the appropriateness of an SABE analysis, considering the fact that the S_{WR} was consistently >0.294 (Table III) and to explore the statistical power of future IVPT BE studies for such products. In order to determine the number of donors that would adequately power such an IVPT BE study, power simulations were performed for both PK parameters (J_{max} and AUC) using an ABE analysis as well as an SABE analysis, and using the BE limits of 0.8–1.25 as well as 0.75–1.33. These power curves, based on 500,000 simulations, are depicted in Figs. 6 and 7.

The more permissive BE limits of 0.75–1.33 were included in the power simulations comparing ABE and SABE only to illustrate that the power (and efficiency) of an IVPT study is increased to a greater magnitude by an SABE statistical analysis of the results than by widening the BE limits for an ABE analysis. Instead, using an SABE analysis when the S_{WR} is >0.294, while maintaining the traditional BE limits of 0.8–1.25, increases the power of the study to an even greater degree than by widening the BE limits to 0.75–1.33 for an ABE analysis. Based, in part, on the results reported here, the FDA determined that the marginal additional power gained by an SABE analysis with more permissive BE limits of 0.75–1.33 was not warranted (15) and, as a result, a SABE analysis with traditional BE limits of 0.8–1.25 was developed and recommended for IVPT studies (16). The more permissive BE limits of 0.75–1.33 are not currently accepted by the FDA, and it should not be inferred from the inclusion of these data in the analysis that the Agency is considering any widening of the BE limits.

Another simulation study evaluated the statistical power as a function of the point estimate, GMR (Figs. 8 and 9). For each PK parameter, the power was determined for a fixed sample size. The results illustrated that for values of the true GMR inside the interval [0.80, 1.25], a power of at least 80% can be achieved with a sample size of 6 or more donors for total AUC (Fig. 8) and 14 or more donors for J_{max} (Fig. 9).

DISCUSSION

In this study, the results of an IVPT study conducted using multiple replicates from 6 donors were presented. The results

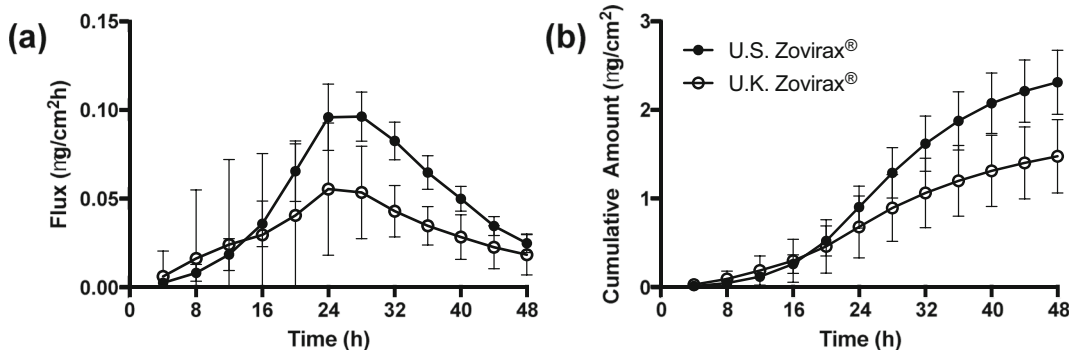

Fig. 2 (**a**) Flux profiles and (**b**) cumulative permeation levels of U.S. Zovirax® and U.K. Zovirax® acyclovir creams (Mean ± SEM of 6 donors, n = 4–7 replicates per donor).

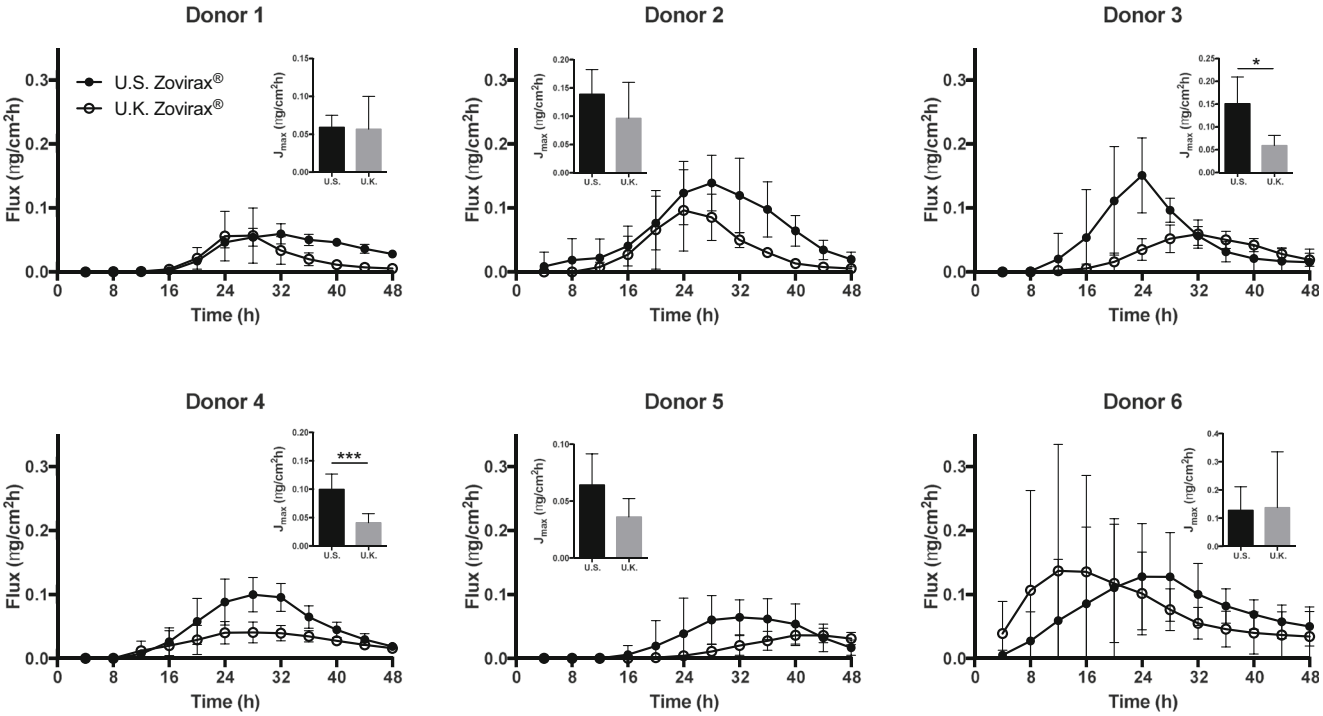

Fig. 3 Comparison of U.S. Zovirax® and U.K. Zovirax® creams per donor (* $p \leq 0.05$; *** $p \leq 0.001$). (Mean ± S.D., $n = 4$–7 replicates per donor).

presented in this work are from a pivotal study that was conducted after evaluating various IVPT experimental conditions, including but not limited to the dose amount, dose application technique, analytical method, and flow-rate of a diffusion cell system in a pilot study. Since the aforementioned factors could influence IVPT results (17–20), it is imperative to conduct a pilot study to determine experimental conditions for a pivotal study based on the goal of the experiment. In addition to the experimental conditions, the study also demonstrated the importance of choosing an appropriate sample size (number of donors and replicates) when conducting an IVPT, especially with drug products anticipated to result in high variability. For example, comparison of J_{max} from two Zovirax® creams in Fig. 3 from individual donors did not always result in a statistically significant difference; however, comparison of J_{max} using collective data from 6 donors in Fig. 4a resulted in a statistically significant difference between the two creams. The present study also evaluated the IVPT results with a statistical approach designed to accommodate the nature of the variability in the data when evaluating the BE of topical dermatological drug products. Despite the large inter- and intra-donor variability observed with IVPT data, the results demonstrated the accuracy, reproducibility, and sensitivity of the IVPT method by confirming the BE of the positive controls (R vs R and T vs T) while discriminating the negative controls (T vs R).

While it is evident from Table I that the four acyclovir cream, 5% products appear to be compositionally different, the amount of each ingredient in these products and how they contribute to the distinct permeation profiles observed (Figs. 1 and 2) are unknown. Trottet *et al.* found that the amount of propylene glycol (PG) in an acyclovir cream and the percutaneous permeation of acyclovir are positively related, with the cream that contained 40% PG delivering 10-fold more acyclovir than the similar formulation containing 15% PG (prepared by substituting 25% PG by 25% water) (21). Consistent

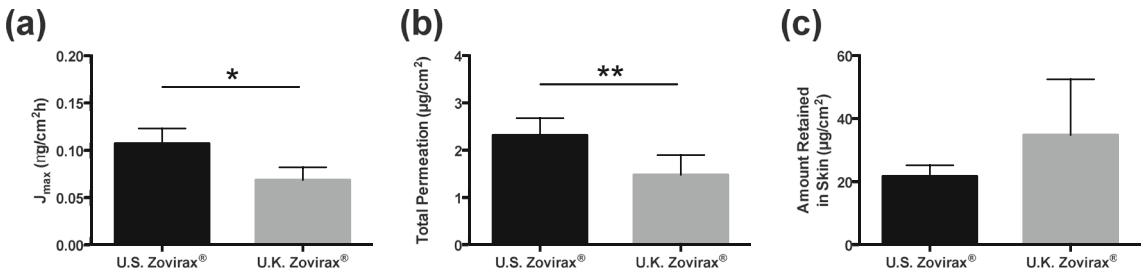

Fig. 4 Comparisons of (**a**) J_{max}, (**b**) the total amount of acyclovir permeated over 48 h and (**c**) amount of acyclovir retained in skin layers after 48 h between U.S. and U.K. Zovirax® creams (* $p \leq 0.05$; ** $p \leq 0.01$). (Mean ± SE, n = 6 donors with 4–7 replicates per donor).

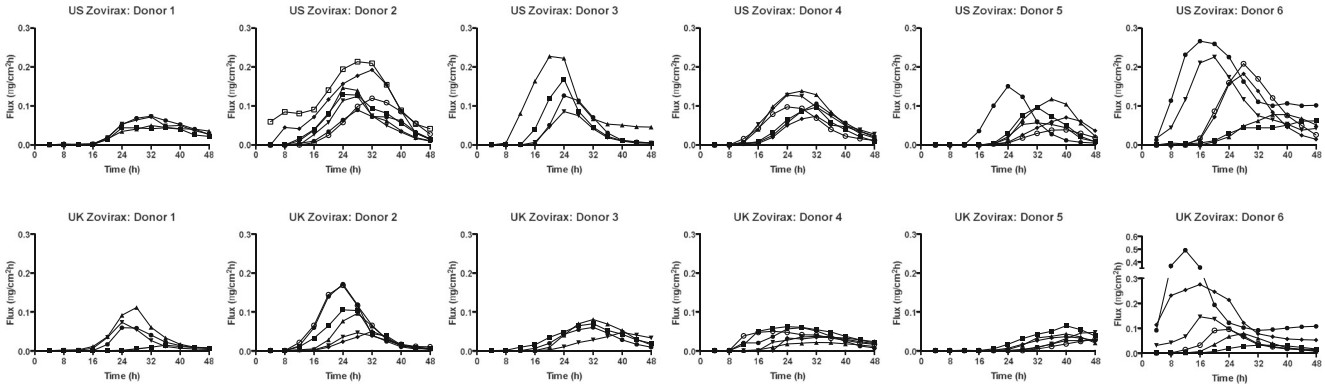

Fig. 5 Flux profiles of U.S. Zovirax® (top) and U.K. Zovirax® (bottom) creams per donor, showing intra-donor variability (n = 4–7 replicates per donor).

with the results from the current study, the U.K. Zovirax® cream provided a much higher permeation over 24 h compared to Aciclostad® cream, with the U.K. Zovirax cream® delivering 6.5 times more acyclovir through the dermatomed skin (21). The same study also determined the PG content in the U.K. Zovirax® and 10 other generic acyclovir creams marketed in European countries. The U.K. Zovirax® contained the highest amount of PG at 40% (*w*/w), and 9 generic creams, including Aciclostad®, contained 14–15% (w/w) PG. One generic cream had no PG content. While it is possible that the Zovirax® and Aciclostad® creams used in the Trottet *et al.* study and those used in the current study are different in terms of formulation and/or manufacturing process, the data from the two studies collectively indicate that formulation differences can result in different permeation profiles with PG playing a significant role, and that an IVPT method was able to detect such differences. We also speculate that the two Zovirax® creams might have higher levels of PG content compared to the two non-Zovirax® creams investigated in the current study.

Compositional differences between products may not only influence acyclovir permeation profiles, but may also have the potential to affect the retention of acyclovir in the skin. Therefore, the amount of acyclovir retained in skin after 48 h was compared between the U.K. Zovirax® and the U.S. Zovirax®. The amount of drug extracted from skin samples must be interpreted with caution, because even with reasonable efforts to clean the skin surface, the amount of acyclovir extracted from the skin samples may include some acyclovir from residual cream that remains associated with the surface furrows, topographical irregularities of desquamation, and invaginations into the appendages of the skin. Assuming that three wipes with an isopropyl alcohol pad is ~97% efficient at removing the dried cream on the surface of the skin, the residual amount of cream associated with the skin surfaces should only be a very small proportion of the applied dose. However, since the amount of acyclovir applied to the surface of the skin (750 $\mu g/cm^2$) is orders of magnitude greater than the total amount (~2 $\mu g/cm^2$) that permeates into the skin, even very small amounts (~3%) of the residual acyclovir cream that remain associated with the outer layers (or structures) of the skin may represent an amount of acyclovir (~22.5 $\mu g/cm^2$) that is still an order of magnitude greater than what permeates through the skin across the entire study duration (which is consistent with the difference in the magnitude of the data plotted in Fig. 4b vs. 4c). Also, relatively small differences in the efficiency of skin cleaning (potentially due to compositional differences between products) could introduce relatively large errors into the apparent amount of acyclovir in (or on) the skin between treatment groups.

Thus, the apparent amount of drug retained in (or on) the skin may not necessarily be an accurate measure of comparative drug permeation. By contrast, J_{max} and total permeation are not only more accurately measured, they are also more directly relevant parameters to assess the rate and extent to which acyclovir becomes available at or near a site of action in the skin. Specifically, J_{max} and total permeation are, therefore, more relevant PK endpoints to support an assessment of BE than the amount of drug apparently retained in the skin, all the more so when the latter is assessed at only a single point in time. Nonetheless, in this study the amount of acyclovir retained in skin after 48 h was compared between the U.K. Zovirax® and the U.S. Zovirax® and no significant difference in skin content was observed between the products (Fig. 4c). By contrast, significant differences between these products were evident for J_{max} (Fig. 4a) and total permeation (Fig. 4b) which suggests that

Table II The *p* value from Brown-Forsynthe Test for Each Donor, Evaluating the Variance of Individual Skin Sections' Flux Profiles from the Same Donor

	Donor 1	Donor 2	Donor 3	Donor 4	Donor 5	Donor 6
U.S. Zovirax®	0.1987 (ns)	0.2926 (ns)	0.3085 (ns)	0.1942 (ns)	0.1203 (ns)	0.0906 (ns)
U.K. Zovirax®	0.0933 (ns)	0.0748 (ns)	0.2307 (ns)	0.1056 (ns)	0.0435 (*)	0.0120 (*)

Table III BE Comparisons of the Test and Reference Products

Product comparison	IVPT PK parameter	Point estimate	Within-reference standard deviation (S_{WR})	SABE upper bound m = 1.25	SABE upper bound m = (1/0.75)	ABE 90% confidence interval
T vs R	Total AUC	0.5314	0.4457	0.5957	0.4993	(0.4208,0.6711)
	J_{max}	0.4926	0.4238	0.9859	0.8950	(0.3459,0.7014)
R vs R	Total AUC	0.9439	0.5032	−0.0864	−0.1629	(0.3390,1.5488)
	J_{max}	0.8339	0.7618	−0.0326	−0.2459	(−0.0887,1.7565)
T vs T	Total AUC	0.9766	0.7132	−0.1894	−0.3409	(0.6724,1.2808)
	J_{max}	0.9966	0.7392	−0.2244	−0.3768	(0.7645,1.2287)

J_{max} and total permeation are not only more relevant and more accurate, but also more sensitive parameters (than the apparent amount of drug retained in the skin) to compare the rate and extent of drug bioavailability from two topical products. Thus, the focus of this study was to evaluate the BE of two products by measuring the permeation of acyclovir through the skin rather than the amount retained in (or on) the skin.

It is important to acknowledge that the current *in vitro* study results cannot support conclusions about the relative clinical (therapeutic) effectiveness of the products evaluated, however, the differences in acyclovir bioavailability observed *in vitro* appear to be in agreement with the results from an *in vivo* dermal open-flow microperfusion (dOFM) study comparing the same products (22). In the dOFM study by Bodenlenz *et al.*, the U.S. Zovirax® and 1A Pharma® acyclovir creams were found to be not bioequivalent, which is in line with the findings from the current study that the two products deliver different amounts of acyclovir (Fig. 1). Interestingly, the differences between the two products seem to be more pronounced in the current IVPT study (based upon flux profiles) compared to the differences observed from Bodenlenz *et al.*'s dOFM study (based upon concentration vs. time profiles), illustrating the exceptional discrimination sensitivity of the IVPT method. The efficiency of the IVPT BE study evaluated with an SABE statistical analysis is evident in the power curves in Figs. 6 and 7 which indicate that, based upon the T vs. R comparison in the current dataset of 6 donors with 4 replicates per donor per treatment

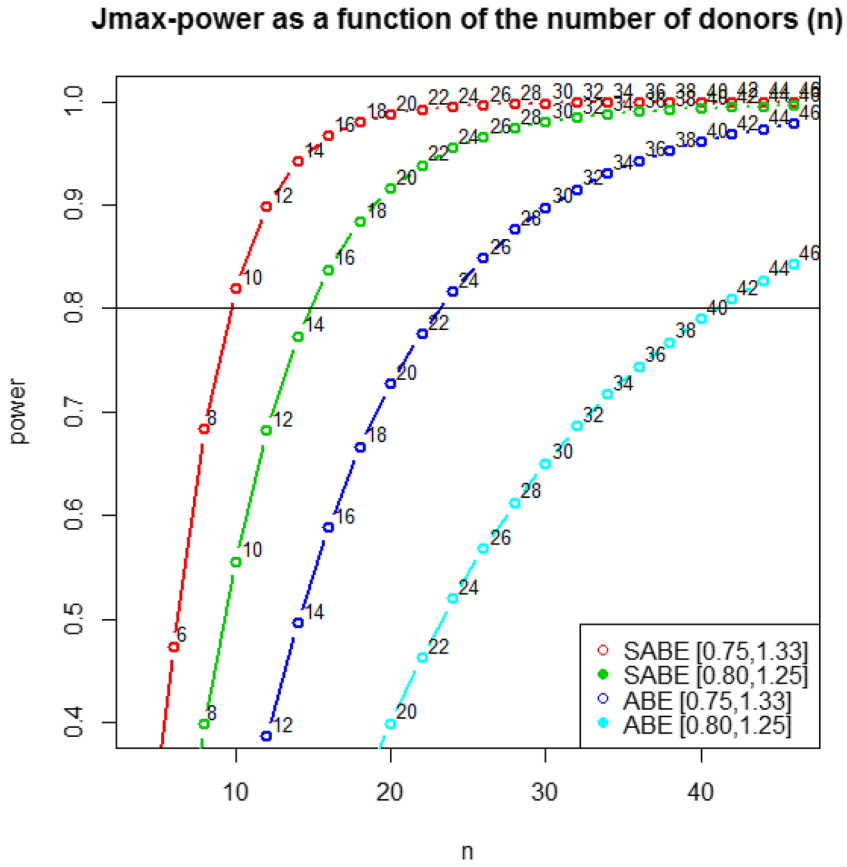

Fig. 6 J_{max}– Power as a function of the number of donors (n).

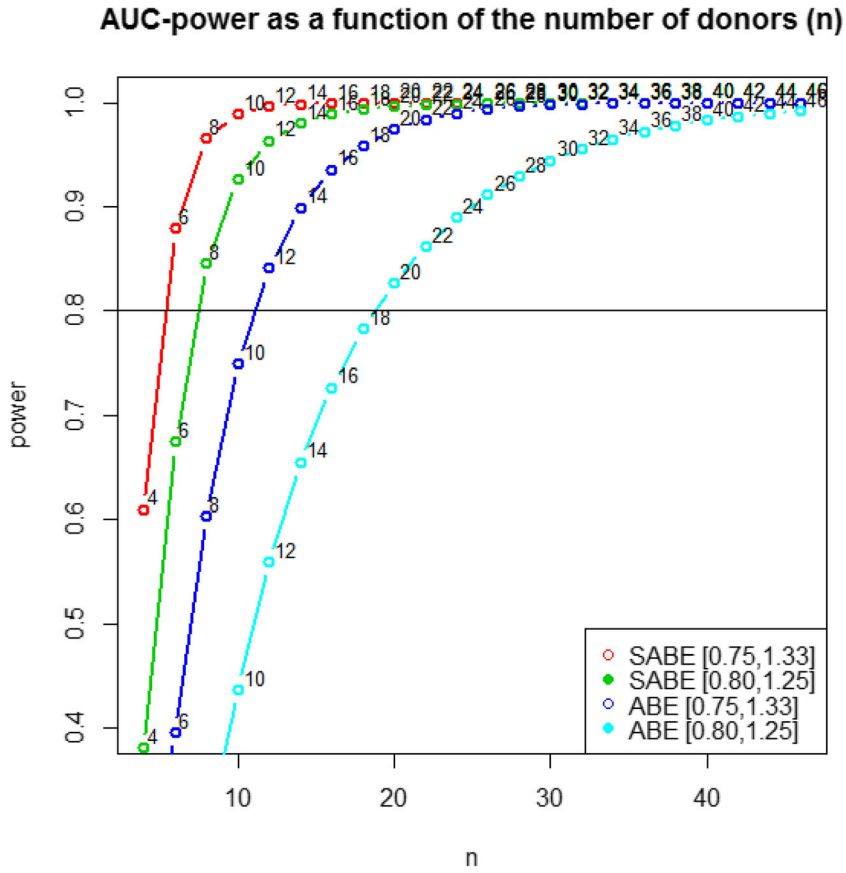

Fig. 7 AUC– Power as a function of the number of donors (n).

group, a minimum power of 80% could be achieved for an IVPT BE study with 16 donors (for J_{max}; only 8 donors would be needed for AUC with BE limits of 0.80–1.25). By comparison, without the SABE analysis, using the traditional ABE analysis, it would likely require approximately 40 donors for the same IVPT BE study design to achieve a minimum power of 80% (Figs. 6 and 7; ABE with BE limits of 0.80–1.25). The latter estimate is similar to the 38 subjects that were estimated to be needed for an *in vivo* dOFM BE study with the same T and R products, in order to achieve a minimum power of 80% using an ABE statistical analysis with BE limits of 0.80–1.25 (22).

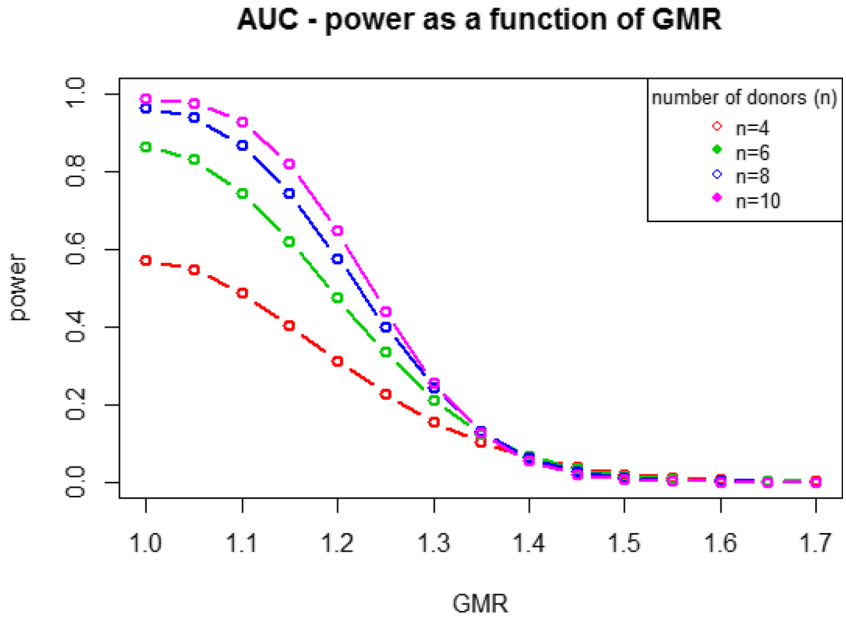

Fig. 8 AUC– Power as a function of GMR.

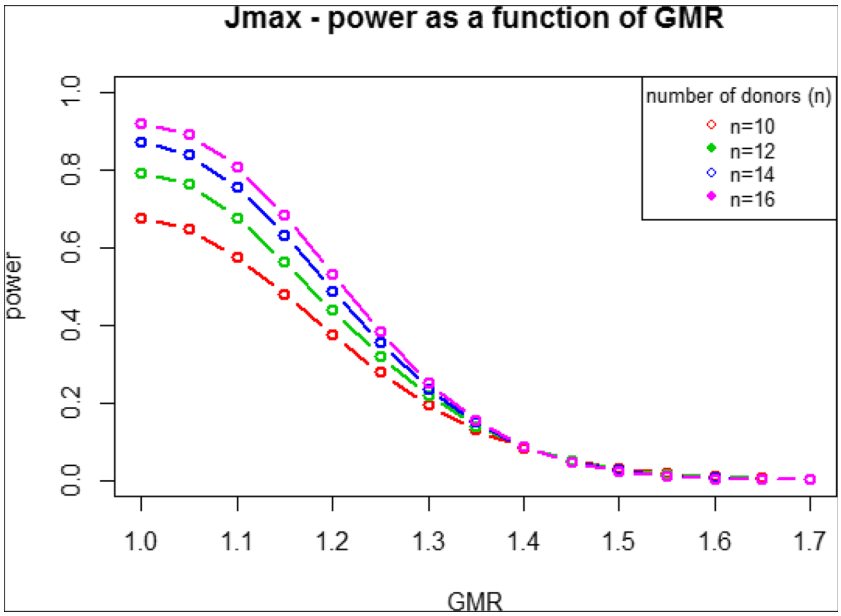

Fig. 9 J_{max}– Power as a function of GMR.

In addition to the *in vivo* dOFM method described above, a SC sampling approach via skin stripping in humans was examined to assess the BE of topical acyclovir products in the recently published work by Pensado *et al.* (23). While the applied dose was left on the skin for the entire duration of the 48 h IVPT study and the 36 h dOFM study, the design of the skin stripping study was fundamentally different inasmuch as the results were based upon a 6 h dose duration, after which the applied dose of each acyclovir cream was removed and the clearance of acyclovir from the SC over the subsequent 17 h was assessed. Despite the differences in study designs, the results from the skin stripping Study 1 suggested that the U.S. and the U.K. Zovirax® creams are not bioequivalent, consistent with the current study's findings. Furthermore, Pensado *et al.* estimated that the average *in vivo* flux of acyclovir from the U.S. Zovirax® was higher than the flux from the U.K. Zovirax®, which is also consistent with the current study's findings.

The skin stripping Study 2 suggested that Aciclovir 1A Pharma® is not bioequivalent to U.S. Zovirax®. This is also consistent with the results of the current study (in which substantial differences in acyclovir bioavailability were observed between Aciclovir 1A Pharma® and U.S. Zovirax®) and consistent with the *in vivo* dOFM study results reported by Bodenlenz *et al.* However, while both the current IVPT study and the *in vivo* dOFM study indicated that the bioavailability of acyclovir from Aciclovir 1A Pharma® was lower than that from U.S. Zovirax® across 36 to 48 h dose durations, the results from the skin stripping Study 2 estimated that the flux of acyclovir from Aciclovir 1A Pharma® is higher than the flux from U.S. Zovirax® based upon a 6-h dose. Considering the fundamental differences in the study design of the skin stripping study compared to the current study, and the fact that the comparisons of the Zovirax® products to non-Zovirax® products, including Aciclovir 1A Pharma®, in the current study were made with limited data from two donors during the initial screening of products, further evaluation is necessary to explain the differences observed between the skin stripping study and the other two studies in relation to Aciclovir 1A Pharma® and U.S. Zovirax®.

When a relatively small sample size (e.g., 16 donors with 4 replicates per donor per treatment group) can be utilized to demonstrate BE, it makes the IVPT method attractive; this is especially true when compared to comparative clinical endpoint studies that were traditionally used to demonstrate BE for topical drug products. Comparative clinical endpoint studies usually require hundreds to thousands of subjects with a long duration of study. Not only is an IVPT study more efficient, but an IVPT method allows for a relatively tight control of critical parameters that may influence drug delivery and the BA of topically administered drugs (which might be difficult to attain in clinical studies). For example, the quantity of dose applied on skin samples in IVPT studies can be precisely controlled without much variation and determined. In the current study, a positive displacement pipette was utilized. While the use of a pipette to deliver and spread the dose on skin surface did not represent an ideal imitation of spreading and rubbing in a clinical, real-use situation, it did allow for a good control of the quantity of dose without much variation in the dose amount applied on different skin samples. Of relevance, the quantity of the dose from semisolid topical products is often not standardized but, rather, determined by the patients in comparative clinical endpoint studies (24,25). Additionally, since the quantity of the dose being applied can vary depending on the disease state, and since the size of the affected surface area with one of the common instructions for dose

application being "apply a thin layer to affected area", comparative clinical endpoint studies in which subjects with varying disease conditions are enrolled have an added challenge.

The burden and inefficiency associated with comparative clinical endpoint BE studies for topical dermatological drug products are well recognized. As a result, alternative approaches by which to demonstrate BE for topical dermatological drug products have now been proposed and/or implemented in the U.S., Europe and elsewhere, supported by evidence from cutaneous PD studies (the vasoconstrictor assay) or cutaneous PK studies (e.g., IVPT and dOFM/ dermal microdialysis). For example, based in part on the research described here, the U.S. FDA has published a draft product-specific guidance on developing a generic version of acyclovir cream, 5% that recommends an *in vitro* option by which to demonstrate BE for acyclovir creams that are Q1 and Q2 the same, with similar physical and structural characteristics, using evidence from an IVPT study, including an SABE analysis of the results (16).

CONCLUSIONS

The current study showed that the use of an IVPT method with an adaptation of the reference scaled statistical approach to evaluating BE was sufficiently sensitive to discriminate compositionally similar, pharmaceutically equivalent cream products as not being bioequivalent, while accurately and reproducibly determining each product to be bioequivalent to itself, even in the presence of significant variability. Furthermore, the implementation of the SABE statistical analysis was shown to be an appropriate approach by which to evaluate BE for this IVPT data based upon the observation that S_{WR} values that were consistently >0.294, and this analysis was shown to improve the power of the IVPT BE study, thereby reducing the number of donors needed to power the study and further improving the efficiency of the study relative to the un-scaled ABE analysis. The current findings indicated that an IVPT method, with an appropriate statistical analysis, can be one of several useful tools to support a demonstration of BE for topical dermatological drug products.

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