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Research Article

Application of an Optimized Tape Stripping Method for the Bioequivalence Assessment of Topical Acyclovir Creams

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Abstract. This study indicates the application of tape stripping (TS) for bioequivalence (BE) assessment of a topical cream product containing 5% acyclovir. A TS method, previously used successfully to assess BE of topical clobetasol propionate and clotrimazole formulations, was used to assess BE of an acyclovir cream (5%) formulation as well as a diluted acyclovir formulation (1.5%) applied to the skin of healthy humans. An appropriate application time was established by conducting a dose duration study using the innovator product, Zovirax® cream. Transepidermal water loss was measured and used to normalize thicknesses between subjects. The area under the curve (AUC) from a plot of amount of acyclovir/strip *vs* cumulative fraction of stratum corneum (SC) removed was calculated for each application site. BE was assessed using Fieller's theorem in accordance with FDA's guidance for assessment of BE of topical corticosteroids. Adco-acyclovir cream (5%) was found to be BE to Zovirax® cream, where the mean test/reference (T/R) ratio of the AUC's was 0.96 and the bioequivalence interval using a 90% confidence interval was 0.91–1.01 with a statistical power >95%, whereas the diluted test product fell outside the BE acceptance criteria with T/R ratio of AUC of 0.23 and a 90% CI of 0.20–0.26. This study indicates that the data resulting from the application of this TS procedure has reinforced the potential for its use to assess BE of topical drug products intended for local action, thereby obviating the necessity to undertake clinical trials in patients.

KEY WORDS: acyclovir cream; topical dosage forms; local action; bioequivalence; acceptance criteria; tape stripping; FDA guidance.

INTRODUCTION

The assessment of bioequivalence (BE) of topical drug products intended for local action, apart from topical corticosteroid formulations (1) and, more recently, several FDA guidance which provides for use of *in vitro* methods for topical acyclovir ointments (2) and creams (3), dapsone gel (4), docosanol cream (5), and ivermectin cream (6), requires comparative clinical trials to be carried out in patients. As clinical end point studies are generally associated with poor discriminatory power and are expensive and also time consuming, the development of techniques to overcome the above shortcomings for determining BE of topical products for local action has provided a daunting challenge which currently remains unresolved.

Although several methodologies to assess BE of topical products intended for local action have been investigated in the past (7–9), and more recently, the application of open flow microperfusion (dOFM) (10), the tape stripping technique has shown the best potential when a draft guidance issued by the US FDA was published in 1998. That draft guidance recommended tape stripping (TS) as a surrogate method for certain classes of drugs, for example, antifungals that target the SC (11), but it was withdrawn in 2002 (12) due to the lack of reproducibility of the method between laboratories.

The tape stripping method was developed in the 1940s, and examined by Pinkus in 1951 (13), to determine drug concentrations in the *Stratum corneum* (SC) by sequential removal of microscopic layers (typically 0.5–1 μm) of the SC (14,15) using strips of adhesive tape applied to the skin surface with uniform pressure, and then systematically and sequentially removed as described by Parffit *et al.* (15), extracted and quantified for drug concentration using a validated analytical method. Several other publications describing the application of tape stripping to assess topical drug

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product bioavailability have appeared in the literature (16–18). Tape stripping is relatively simple, inexpensive, and has been described as a minimally invasive technique (19).

Au *et al.* (20) have illustrated the potential of a standardized TS method by demonstrating the application of TS to assess the BE of two 0.05% clobetasol propionate cream formulations. Subsequently, an improved TS method described by Parfitt *et al.* (15) was used to successfully demonstrate the BE of topical clotrimazole formulations. These authors emphasized the need to optimize the method by determining an appropriate dose duration as well as to standardize skin thickness between subjects to enhance the sensitivity, precision, and reproducibility of the TS technique.

Although the SC is thought to be the major rate determining step for the passage of most molecules across skin (21), but further penetration beyond and into the dermis and deeper layers may also be hindered by the viable epidermis as well as influenced by the physicochemical properties of the drug (22). Additionally, since corneocytes and intercellular lipids are responsible for preventing insensible water loss, the transepidermal water loss (TEWL) can be measured and the data can be used to assess skin barrier integrity. TEWL increases as a function of tape strip number and depends on factors such as, anatomical site, pressure, pressure duration, and tape removal rate. Furthermore, it has also been demonstrated that TEWL increased fastest on the forehead, followed by the back, and finally, the forearm. These findings were purported to explain the differences in SC thickness, differences in spontaneous desquamation (SC cohesion), and pressure resistance because of inherent viscoelasticity and type of tissue underlying the skin (23). Based on the improved TS procedure described by Parffit *et al.* (15), the BE of a generic 5% acyclovir cream (Adco-acyclovir cream) as well as a 1.5% acyclovir cream was investigated by applying that method.

METHODS AND MATERIALS

Chemicals

ACV reference standard (RS) was purchased from Sigma-Aldrich Co., (St. Louis, USA) and HPLC grade methanol (UV cutoff 215 nm) was purchased from Romil Ltd. (Cambridge, UK). HPLC grade water was generated in a MilliQSystem (Millipore, Milford, CT, USA).

HPLC Method

The HPLC system consisted of a Luna 5 μm C8(2) 150 × 4.60 mm (Phenomenex) column perfused at a flow rate of 1 ml/min by a mobile phase consisting of 0.1% aqueous formic acid and methanol in the ratio 95:5. The ACV peaks were monitored at a wavelength of 254 nm.

Extraction Procedure

Each tape strip (Scotch® Magic Tape) of approximately 2 × 2 cm dimensions was placed into a 1.5 ml polypropylene centrifuge tube with the adhesive side of the tape facing towards the inside of the tube. Each tape was extracted with 500 μl of water for 15 min at 60 °C, and vortexed for 1 min (twice) using the Eppendorf MixMate vortex mixer (Eppendorf Ag, Hamburg, Germany). Approximately 100 μl of the sample was pipetted into a microinsert that was placed into an HPLC amber vial and the samples were analyzed using Shimadzu UFLC.

Each tape strip was spiked with 10 μl of a standard solution and left to air dry. The tape strips were then placed into a microcentrifuge and extracted according to the procedure mentioned above. After extracting the spiked tape strips, the final concentration range was 0.01–10 μg/ml (10, 5, 2.5, 1.0, 0.5, 0.25, 0.1, 0.05, 0.025, and 0.01 μg/ml).

A fresh, separate stock solution was used to prepare the quality control (QC) samples on a daily basis. Three QC standard solutions of 100, 50, and 25 μg/ml (high, medium, and low concentrations) were extracted to give final concentrations of 2, 1, and 0.5 μg/ml.

Formulations

The studies involved the use of two commercially available products containing 5% acyclovir. Zovirax® is the innovator brand of ACV, and therefore, Zovirax® Cream, manufactured by GlaxoSmithKline South Africa (Pty) Ltd., was used as the reference product/reference listed drug (RLD). Adco-acyclovir topical cream manufactured by Adcock-Ingram (Pty) Ltd., SA, was used as the test product. In addition, the placebo cream base was obtained from Adcock-Ingram (Pty) Ltd., SA, which was used to extemporaneously dilute the 5% Adco-acyclovir topical cream to obtain a lower concentration of 1.5% acyclovir cream for testing in the BE study.

Study Design

A dose duration study in accordance with the procedures and processing described by Parfitt *et al.* (15) was employed to determine a suitable dose duration for the BE study. BE studies were subsequently conducted using a dose duration equal to the ED_{50} determined from the E_{max} model. The 6-point dose-response profile was constructed using mean AUC values obtained for each dose duration ($n = 6$). An AUC value of 0 was assumed at time = 0 min as no ACV penetration could have occurred. This 6-point profile was fitted to the E_{max} model and the ED_{50} calculated using GraphPad Prism software version 4 (GraphPad Software, San Diego, California, USA). For the bioequivalence investigations, Adco-acyclovir topical cream was used as the test (T) product and Zovirax®cream was used as reference (R) product to assess BE. Additionally, in a separate study intended to show non-bioequivalence (bioinequivalence), Adco-acyclovir topical cream was diluted with placebo base to result in a 1.5% acyclovir content and compared with the same reference product, Zovirax® cream used in the previous study.

Six volunteers (four male, two females) between the ages of 19 and 28 (mean 22 years) of Indian origin were enrolled in the dose duration study. Written informed consent was obtained from each volunteer before each study. The research with human subjects followed the recommended guidelines as set out in the Declaration of Helsinki (1964) and associated amendments (24). The study protocol was

approved by the KLE University Ethical Standards Committee (Belgaum, India).

Six sites (2 × 2 cm) were demarcated on the left arm of each subject (Fig. 1) using a template prepared by reinforcing the non-adhesive side of Opsite Flexifix (Smith & Nephew Medical Ltd., London, England) with ScotchMagic™ Tape (No.810, 3M, USA). Five of the sites were used for product application and one site was reserved as a blank site. An amount equivalent to about of 20 mg acyclovir topical cream using a calibrated Eppendorf pipette was dispensed to each site at time zero. A pre-weighed glass rod was used to spread the product within the area delineated by the template and the rod was weighed following spreading.

Each application site was exposed to the formulation for different dose durations (2, 4, 8, 15, and 30 min). Once the necessary dose duration had elapsed, three cotton swabs were used to remove the residual formulation from the site and the TS procedure commenced. The tape stripping procedure involved the use of 15 pre-weighed tape strips per site. The tape strips were prepared in-house using ScotchMagic™ Tape. The direction of stripping was rotated (N, W, S, and E) as previously described to ensure uniform removal of SC (15). After stripping, each tape strip was immediately weighed using a Shimadzu analytical balance AUW220D (Shimadzu Corporation, Kyoto, Japan). The use of 15 tape strips consistently removed an average of 78.67 ± 5.16% (mean ± SD, $n = 30$) of the SC from the application sites. Typically, masses of SC removed were found to be in the range 0.1–0.8 mg.

The blank site underwent the same TS procedure, but in addition, TEWL measurements were taken after each stripping using a Delfin VapoMeter (Delfin Technologies Ltd., Finland) in order that the thickness of each subject's SC could be determined. Untreated skin samples from blank sites were spiked with standard solutions of ACV to generate calibration curves analyzed using HPLC to determine ACV concentrations. The tape strips from both the blank and drug application sites were extracted as previously described and analyzed.

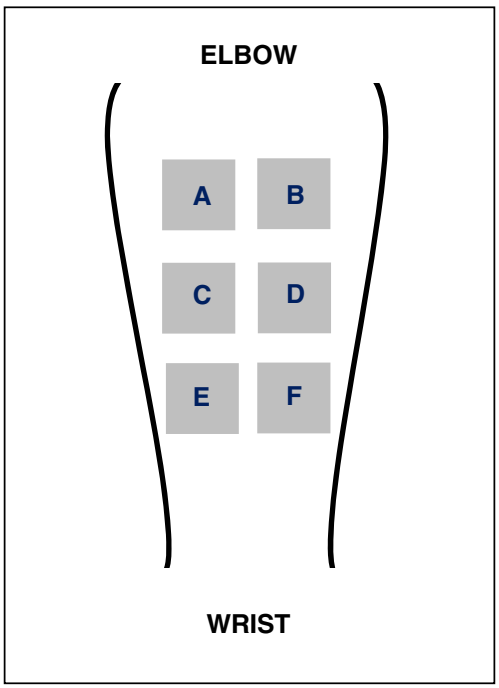

Fig. 1. Template used for TS study in study SN_TS 1

Whereas, the SC thickness can be determined using the SC assay described by Dreher (25), the SC thickness (H) for each individual was determined as previously described using the TEWL measurements (13). This involved the use of the following relationship,

$$\frac{1}{TEWLx} = \frac{H}{K\Delta C \cdot D} - \frac{x}{K\Delta C \cdot D}$$

TEWLx represents the transepidermal water flux when *x* mm of SC has been removed by tape stripping, *K* describes the SC-viable tissue partition coefficient of water, *D* is the average apparent diffusivity of water, ΔC is the water concentration difference across the membrane, and *H* (μm) is the total thickness of the SC. Since *K*, ΔC, and *D* are constant, this relationship can be further simplified as $1/TEWLx = -x + H$. Assuming that the SC adhering to each tape strip is uniform and has a density of 1 g/cm^3 (14), *x* can be calculated from the SC mass. Therefore, *H* can be determined from the *x*-intercept of the plot $1/TEWLx$ *vs x*.

The total SC thickness for each individual was used to correct for differences in SC thickness between subjects and to normalize the data. As previously explained (15), the first tape strip from each application site was not included in the data analysis since it may have contained some unabsorbed drug product remaining on the application site on the skin that had not been completely removed by the cotton wool swabs.

AUC values were obtained from a plot of the amount of drug per strip *vs* x/H (cumulative fraction of *SC* removed) and calculated for each application site using the trapezoidal rule. The AUC values were used as the BE metric such as used in the FDA's Vasoconstrictor Assay guidance (VCA) (20), and also as previously successfully applied to assess the BE of topical products (15,20) together with appropriate statistical procedures and regulatory acceptance criteria.

Furthermore, the use of AUC as the preferred metric (17) to determine bioequivalence for topical products for local action as opposed to using both the rate (C_{max}) and extent (AUC) as is the case for drugs intended for the systemic circulation is an accepted parameter and used in the FDAs VCA assay and is also described in the Japanese guidelines for topical products (26).

Bioequivalence Studies

Two *in vivo* TS studies were undertaken to assess bioequivalence where the first study involved a generic acyclovir cream containing 5% acyclovir (Adco-acyclovir topical cream) and the second study using an extemporaneously diluted Adco-acyclovir topical cream containing 1.5% acyclovir. The same innovator product, Zovirax® Cream, was used as the reference product in both studies.

The first bioequivalence study was conducted using 20 subjects (6 females and 14 males) between the ages of 19 and 30 years (mean 25 years) from Indian descent. As previously described (15), five (2 × 2 cm) sampling sites on the volar

aspect of the left forearm were demarcated using a template. One of the sites was assigned as a blank, two of the sites were randomly designated as "test" sites, and the other two as "reference" sites for product application. Approximately 20 mg (accurately dispensed from a calibrated dispenser) of acyclovir topical cream was applied to each application site at time zero. After the required dose duration had elapsed, the residual formulation was removed using three cotton swabs per site. The swabs were used to wipe the area using small circular motions and care was taken to remove all the formulation visible to the eye. Tape stripping was subsequently initiated by placing a tape strip on the demarcated site and uniform pressure was applied by rubbing the strip ten times in both an upward and downward motion. The strip was removed with a single upward pull and the direction of stripping is rotated to ensure uniform removal of SC as previously described.

TEWL measurements were taken at the blank site only to calculate the thickness of each subject's SC using a Delfin VapoMeter® (Delfin Technologies Ltd., Finland). After the removal of each tape strip, the VapoMeter® was placed vertically on the site without delay and readings were taken for 15 successive strips.

A second *in vivo* TS study was conducted as a "proof of concept" study to assess whether this TS method could also discriminate between two purposely chosen non-equivalent acyclovir cream products. The same study design was used as previously described where a clotrimazole topical gel product was compared with the innovator product, Canesten Topical cream used as the reference (15) where bioinequivalence was successfully demonstrated. Ten healthy subjects (two females and eight males) between the ages of 19 and 30 years (mean 25 years) from Indian descent were included. Zovirax® Topical cream was used as the reference product and compared with a diluted Adco-acyclovir topical cream (1.5%) used as the test product.

An AUC_{test} and $AUC_{reference}$ value was determined for each subject by taking the mean ($n = 2$) of the AUC values for each "product". As each subject received both the test and the reference product, a randomized paired/crossover study design was utilized. The mean $AUC_{test}/AUC_{reference}$ ratios were calculated by dividing the mean AUC_{test} value by the mean $AUC_{reference}$ value. The 90% confidence intervals ($CI_{90\%}$) for the $AUC_{test}/AUC_{reference}$ ratios for both sets of data were determined using Fieller's/Locke's method as described in the FDA guidance (1) using EquivTest 2.0 (Statistical solutions Ltd., Ireland) software and the point estimates were calculated by dividing the mean AUC_{test} values by the mean $AUC_{reference}$ values. The CV% associated with the ratios was calculated as previously described from the following equation,

$$CV\% = \frac{\sqrt{MSE}}{mean} \times 100$$

In order to determine the number of subjects required for 80% statistical power, the method described for "raw data from a cross over study design" by Chow and Wang (27) was used.

RESULTS

HPLC Method Validation

Linearity

The linearity data ($n = 3$) was evaluated by linear regression analysis over the concentration range of 0.01–10 μg/ml. The spiked tape strip extracts gave a linear response with a correlation coefficient of 0.9988 ($y = 31{,}838\times + 124.96$).

Accuracy and Precision

The accuracy and inter- and intra-day precision of the extraction method was assessed using the same low (0.5 μg/ml), medium (1 μg/ml), and high (2 μg/ml) QC standard tape strip extracts ($n = 5$). The accuracy of the method was found to be in the range of 99.9–105.4% with a RSD of < 3% and inter- and intra-day precision was < 4 and < 3.7%, respectively.

Limits of Quantitation and Detection

The limits of detection (LOD) and limits of quantification (LOQ) values were found to be 0.005 and 0.01 μg/ml based upon signal to noise ratios of 3:1 and 10:1, respectively.

Specificity

No interferences were observed following extraction of blank tape strips and tape strips with adhered SC using Scotch® tape.

Recovery

The extraction recovery of acyclovir from the spiked tape strips (high ~ 2 μg/ml, medium ~ 1.0 μg/ml, and low ~ 0.5 μg/ml) was found to be in the range of 86.3–94.4%.

Dose Duration Study

The data were fitted to the E_{max} model with ED_{50} = 9.46 min and $E_{max} = 126.9$ (Fig. 2).

The ED_{50} is considered the time at which maximum sensitivity can be achieved. A dose duration of 8 min was selected in accordance with FDA's topical corticosteroid guideline (1), where D1 corresponds to one half of the ED_{50} (4.73 min) and D2 is two times the ED_{50} (18.92 min); the observed ED_{50} (9.46 min) value was rounded by 8 min which is within the tolerance limits of D1 and D2, also, 8 min falls on the slightly steeper part of the curve in accordance with the appropriate sensitivity.

Bioequivalence Studies

Inspection of the data from the comparative study between the generic Adco-acyclovir cream and the innovator Zovirax® cream indicated that the products can be considered to be bioequivalent based on the acceptance criteria for the study. Mean AUC values of test (Adco-Acyclovir, Adcock Ingram) and reference (Zovirax®, GSK) acyclovir

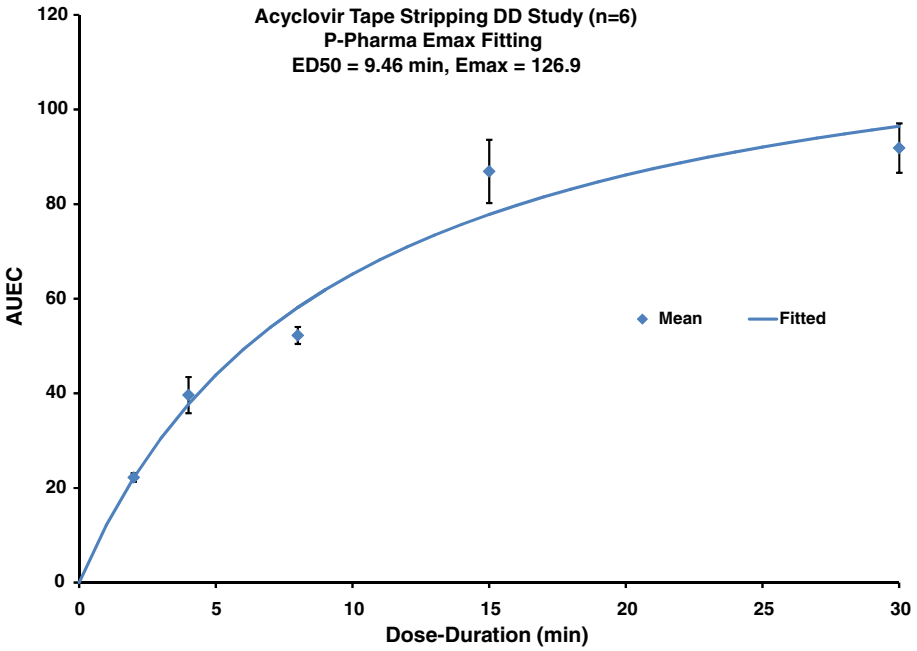

Fig. 2. Dose duration profile for acyclovir (Zovirax®) cream ($n = 6$)

cream formulations using tape stripping were calculated and shown in Table I. The AUC ratio found by comparing the test product Adco-acyclovir topical cream with innovator product Zovirax®cream (*i.e.*, T/R) was found to be 0.96 and with 90% CI of 0.91–1.01 which is within acceptance criteria limits of 0.8–1.25. Furthermore, the inclusion of 20 subjects indicated a statistical power of over 95% and based on a statistical power of 80%, only 12 subjects would be sufficient to confirm BE (Table I).

In the subsequent study comparing the generic product, Adco-acyclovir cream which had been extemporaneously diluted to contain a diluted concentration of acyclovir (1.5%) cream to ensure bioinequivalence was then compared with the reference product Zovirax® cream 5% acyclovir. The AUC ratio T/R of the diluted generic product *vs* Zovirax® was found to be 0.23 with a 90% CI of 0.2–0.26 (Table II), indicating bioinequivalence.

This clearly indicates that the rate and extent of ACV penetration from the undiluted Zovirax® cream was far greater than that of the diluted Adco-acyclovir cream formulation. Although, skin penetration profiles for each product were obtained, since all points have different x/H values, true mean profiles cannot be determined. These mean skin penetration profiles (Figs. 3 and 4) are therefore simply approximate comparative representations of the various drug product profiles within the skin and are useful for visualization purposes only. Data points for these profiles were determined by calculating the mean of both the x values (x/H) and y values (amount per strip) for all subjects for data points with x values within specified sequential ranges over the full x/H range of 0 to 1.

Table I. Summary of BE results—Test *vs* Reference Cream

Parameters	T/R
n (number of subjects)	20
$AUC_{test}/AUC_{reference}$	0.96
$CI_{90}\%$	0.91–1.01
Bioequivalent? (0.8–1.25)	Yes
CV%	14.9%
Power	95.4%
n required for 80% power	12

DISCUSSION

The purpose of carrying out a dose duration study is to determine the length of time that the dose should be left on the skin in order to ensure requisite discriminatory capability of the BE method (15). Hence, sampling when the concentration of drug in the SC is at steady state is likely to mask differences between formulations should such differences in BE exist and it is thus necessary to use a validated method to choose that an appropriate dose duration falls on the sensitive part of a dose–response relationship as shown by a plot of the dose duration *vs* drug penetration profile. The FDA human skin blanching assay (HSBA) guidance (1) was used to determine an appropriate dose duration of 8 min from fitting the data to the E_{max} model. It is also necessary to emphasize that in view of the differences in

Table II. Summary of BE results—Test (diluted) *vs* Reference

Parameters	T/R
n (number of subjects)	10
$AUC_{test}/AUC_{reference}$	0.23
$CI_{90\%}$	0.20–0.26
Bioequivalence? (0.8–1.25)	NO
% CV	24.4%

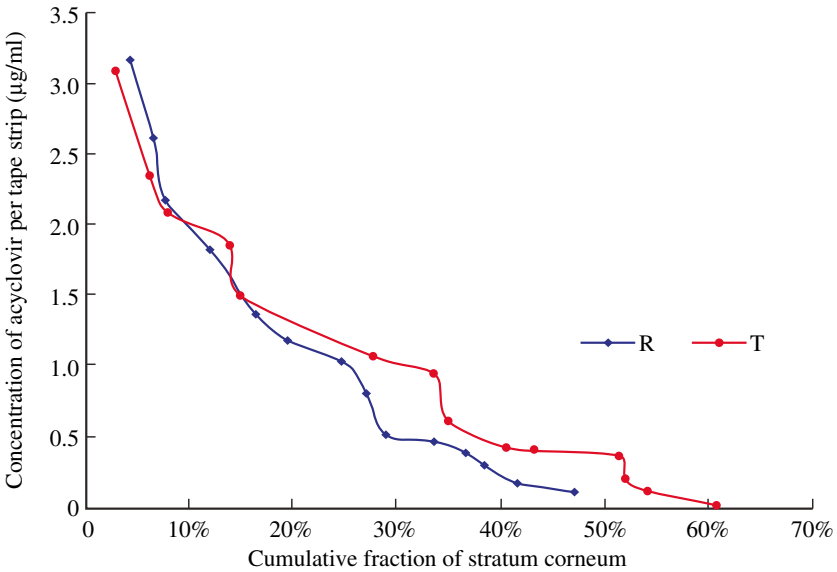

Fig. 3. Comparative skin penetration profiles for test and reference (Zovirax®) creams

skin thickness between subjects, it is important to normalize those data using TEWL measurements (15).

The diluted generic product which contained 30% of the RLD resulted in an AUC ratio T/R of 0.23 and the associated 90% CI of 0.20–0.26 fell outside the BE acceptance limits. This indicates the potential of this technique for use as a surrogate method for the assessment of BE of topical products intended for local action. Further studies, however, are necessary to confirm the discriminatory power to detect smaller differences between a T and RLD.

Apart from the TS method described in the Japanese guidelines (26), which is similar to the method use in the current study, to our knowledge, there are currently no other established regulatory guidelines which make provision for the use of TS and the associated acceptance criteria to assess BE of topical products for local action. The BE limits used in this study (*i.e.,* 0.8–1.25) were based on those described in the FDA guidance used for bioequivalence studies involving oral products (28). The results of this study therefore reinforce previously published TS results using this method for the assessment of BE of topical clobetasol propionate formulations (20) and clotrimazole products (15), thereby providing compelling evidence for the method's potential application to determine BE of topically applied acyclovir creams based on the conventional BE limits. Furthermore, this study, as in previously published studies (15,20), was able to demonstrate that this TS method is also capable of detecting both differences and similarities between ACV cream formulations.

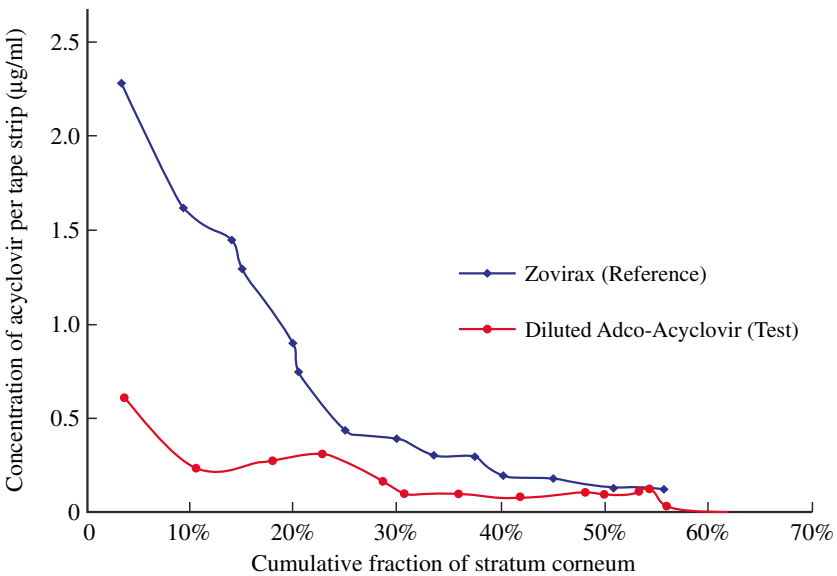

Fig. 4. Comparative skin penetration profiles for test (diluted) and reference (Zovirax®) creams

CONCLUSION

Whereas the inclusion of 20 subjects in the bioequivalence study indicated a statistical power of over 90% to confirm BE, the study involving the diluted product only involved the use of ten subjects where a minimum of 12 would have been necessary to confirm the bioinequivalence findings. Notwithstanding, however, these studies indicate the potential of this improved TS method for use to assess BE between topical ACV products and reinforce previous findings using this optimized TS method and techniques (15,20).The current ACV results provide further evidence of the appropriateness of this technique for application for BE assessment that TS method could be considered as a surrogate procedure and alternative method to clinical studies for the assessment of BE for topical products intended for local action.

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