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RESEARCH ARTICLE

Topical bioequivalence of acyclovir creams using dermal microdialysis in pigs: a new model to evaluate bioequivalence for topical formulations

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Abstract
The aim was to evaluate the bioequivalence of topically applied Acyclovir (ACV) creams using dermal microdialysis (DMD) in a pig model. Three ACV creams (3%), ACV1, ACV2 and ACV3, were topically administrated on the dorsum of pigs, and the DMD sampling technique was used to continuously collect microdialysate. The concentration of ACV in microdialysate was measured by HPLC and the concentration-time profiles were used to calculate pharmacokinetic parameters. The results showed that 90% confidence interval (CI) of the ratio of $AUC_{0-4\ h}$ of ACV2 and ACV3 was between 88.2 and 105.7%, which was within the acceptance range (80–125%). Ninety percent CI of the ratio of C_{max} of ACV2 and ACV3 was between 87.4 and 124.4%, which was within the acceptance range (80–125%). These data indicate that ACV2 and ACV3 used in this study were bioequivalent. This study demonstrates that the pig model coupled with DMD sampling can potentially provide a cost-effective strategy to evaluate topical drug delivery and its associated pharmacokinetic studies.

Keywords: Microdialysis, bioequivalence, acyclovir (ACV), topical formulation, pig

Introduction

Topical bioequivalence is important to determine the *in vivo* therapeutic equivalence of generic and reference topical formulations that contain the same active ingredient. The bioavailability of these formulations when applied at the same dosage is expected to be at a comparable level to reach topical bioequivalence. However, the topical bioavailability is affected by the properties of active ingredient such as the saturation degree, the solubility in vehicle excipients, and/or the diffusivity through the stratum corneum[1–3]. For example, lidocaine in microemulsion showed more than 4-fold increase in cutaneous penetration (*i.e.*, topical drug delivery) than its counterpart in cream, most likely due to the enhanced solubility and diffusivity of lidocaine in microemulsion[4]. Similarly, cutaneous penetration of lidocaine was observed to increase 3–5-fold in cream than in ointment[5]. Due to the use of different vehicle excipients, topical corticosteroid[6], topical ibuprofen formulations[7] and acyclovir creams[8] have also shown varying bioavailability profiles. Hence, there is still an unmet need for bioavailability investigation and bioequivalence assessment to maintain equivalent therapeutic efficacy during the development of generic formulations.

Currently, skin-stripping and clinical evaluations are commonly employed to assess topical bioequivalence. The skin-stripping method, also referred to the dermatopharmacokinetic (DPK) method, involves repeated application and removal of adhesive on the skin to collect consecutive layers of stratum corneum

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for analysis of absorbed drug concentration[9–11]. Despite the simple procedure, this method can hardly be used in diseased skin. In addition, the DPK approach is an endpoint analytical method, which cannot be used to monitor the real-time drug concentration profile[5,12]. Another approach to assessing bioequivalence is clinical evaluation. For example, skin blanching as a clinical indicator has been approved by the Food and Drug Administration (FDA) to evaluate the bioequivalence of corticosteroid formulations that can cause vasoconstriction[13]. Although clinical evaluation is feasible to determine bioequivalence, it is costly and time consuming[14]. Thus, a live sampling technique which enables real-time evaluation of the drug concentration in the skin is highly preferable for topical bioequivalence assessment.

In the last decades, dermal microdialysis (DMD) has been used as a means of continuous sampling to monitor cutaneous drug absorption[15–25]. In this method, an ultrathin, semipermeable probe is inserted in the dermis, and is perfused with a buffer to collect diffused drugs through a semipermeable membrane. The collected drugs in microdialysate can be analyzed by High-performance liquid chromatography (HPLC) or mass spectrophotometry to evaluate spatial distribution of applied drugs. Compared to skin-stripping and clinical evaluation, this technology offers several advantages. First, DMD can provide real-time drug concentration profiles to closely study the delivery of topically applied drugs, whereas skin stripping and clinical evaluation can only provide end-point assessment. Second, DMD is considered as minimally invasive and it has been successfully evaluated in clinical trials[4,26]. Third, DMD can also be applied to diseased skin[27]. Thus, DMD offers a promising alternative to study cutaneous delivery of topically applied drugs in a real-time manner, which is suitable to study topical bioequivalence.

Herein, we report the application of DMD for assessing topical bioequivalence of acyclovir (ACV) creams in pigs. Although DMD has been safely evaluated in human, the testing is costly and the safety issue such as skin irritation remains. As we have reported previously, DMD can also be easily established on the pig skin which is physiologically comparable to human skin[28]. The use of DMD in pigs provides a cost-effective module that is suitable to evaluate topical bioequivalence in a pilot study prior to clinical trial, and holds great potential as an animal model to screen for effective drug candidates for topical treatment.

Methods

Chemicals and reagents

Acyclovir standard (purity of 99.6%) was purchased from Hubei Institute for Food and Drug Control (Wuhan, China). Due to the availability, three ACV (3%) cream formulations (ACV1, ACV2, and ACV3), as opposed to 5% ACV formulations (*e.g.,* Zovirax®) in the EU market, were purchased from a local pharmacy (Nanjing, China). ACV1 contained glycerol monostearate, glycerol, albolene, sodium dodecyl sulfate, liquid paraffin, triethanolamine and ethylparaben. ACV2 contained glycerol monostearate, glycerol, albolene, sodium dodecyl sulfate, octadecanol and benzencarbinol. ACV3 contained glycerol monostearate, glycerol, albolene, sodium dodecyl sulfate, liquid paraffin, simethicone and ethylparaben. The concentration of each component in these ACV formulations was not available from the manufacturers. 3M Tegaderm Transparent Dressings were purchased from 3M Corp. (Methuen, MA, USA). Normal saline was obtained from Xiaoying Pharmaceutical Factory (Nanjing, China). Methanol of liquid chromatographic grade was obtained from Hanbang Corp. (Nanjing, China). Triple distilled water was filtered using 0.45 μm disposable filters (3M Corp.) and was then used to prepare solutions.

HPLC assay

The concentrations of ACV were analyzed using HPLC. The chromatographic system consisted of a chromatographic pump, an injector valve, a 20 μL sample loop and a 2487 ultraviolet detector (Waters Corp, Milford, MA, USA). The samples were loaded onto a reverse-phase column (Lichrospher-C18, 4 μm, 3.9×250 mm, Hanbang Corp. China) and ACV was separated by the HPLC system. The separated ACV was subsequently detected at a wavelength of 254 nm. To facilitate the separation, a mobile phase consisted of water and methanol (95:5, v/v) was used at a flow rate of 1.0 mL/min. The retention time for ACV was 7.8 min and the peak area was highly correlated ($r^2 > 0.99$) with ACV concentrations ranging from 0.02 to 4.0 μg/mL.

In vitro drug release

In vitro drug release of ACV was investigated using a ZRS4 dissolution test apparatus (Tianjin TDTF Technology Co., Tianjin, China). This apparatus consisted of a small glass beaker with an open area of 1.77 cm^2, a 1-L glass beaker and a water bath. The open area of the small glass beaker was wrapped with a cellulose acetate membrane (with a pore size 0.45 μm), on which 0.2 g ACV cream was evenly applied. The small glass beaker was covered with the membrane and inversely placed just in contact with normal saline (900 mL) in the 1-L glass beaker. This setup allowed continuous drug release across the membrane into the normal saline. During the drug release up to 6 h, the normal saline in the 1-L glass beaker was continuously stirred at a rate of 80 rpm and was kept at 37°C in the water bath. Every hour, 1 mL of the normal saline containing the released ACV was collected using a syringe and was replaced with 1 mL of fresh normal saline. The collected saline samples were filtered (0.45 μm) and the drug concentration was quantified using the HPLC method described above.

In vitro microdialysis

Prior to investigation of drug release *in vivo*, the microdialysis system was calibrated by a retrodialysis method

as previously reported[28]. The used microdialysis system consisted of a microinjection pump controller, a microdialysis syringe pump, gastight syringes, and LM-10 linear microdialysis probes with a semipermeable membrane of 10 mm in length and a molecular weight cutoff of 20 kDa (Bioanalytical Systems Inc, West Lafayette, IN, USA). We carried out *in vitro* retrodialysis for calibration before *in vivo* retrodialysis experiments. To this end, we first investigated the effect of flow rate on the recovery and delivery of probes. For *in vitro* recovery measurement, the window of probe was immersed in 1.0 μg/mL ACV solution (or normal saline for delivery measurement) and perfused with normal saline (or 1.0 μg/mL ACV solution for delivery measurement) at a flow rate of 1, 2 and 4 μL/min for 90 min. Then the effect of drug concentration was studied. For measuring the recovery efficiency, the window of the microdialysis probe was immersed in 0.2, 1.0, and 5.0 μg/mL ACV solution and perfused with normal saline at a flow rate of 1 μL/min for 90 min. For measuring the delivery efficiency, the window of the microdialysis probe was immersed in normal saline and perfused with 0.2, 1.0, and 5.0 μg/mL ACV solution at a flow rate of 1 μL/min for 90 min. In both cases, the dialysate was collected every 30 min and subject to HPLC analysis.

In vivo microdialysis

Six Suzhong line 1 pigs (20–30 days old, weighing 5.5–6.5 kg, Jiangsu Academy of Agricultural Science, China) were used throughout the *in vivo* study. All animal experiments were performed according to the requirements of the National Act on the Use of Experimental Animals (People's Republic of China). Pigs were anaesthetized with 30% urethane (intraperitoneal injection, 3 mL/kg) 30 min before each experiment. The dorsum of the pig was shaved carefully with an electrical hair clipper. After shaving, there was a 30-min recovery period. Three linear microdialysis probes were then inserted into the dermis with introducer needles, parallel to the skin on the dorsum (Figure 1). The surrounding areas at the probe inlet and outlet were sealed with 3M Tegaderm Transparent Dressing.

Probes were kept on site for 90 min, allowing for release of the insertion microtrauma. After the equilibrium period, the depths of probes in the dermis were determined by using an SA-6000CMT Doppler Scanner (Medison Medical Instrument Corporation, Shanghai, China). The probes were then perfused with 1.0 μg/mL of ACV solution at a flow rate of 1.0 μL/min for 60 min. Microdialysate samples were collected in duplicate to calculate *in vivo* recovery by Equation 1.

$$\text{Recovery} = (1 - C_{\text{dialysate}}/C_{\text{perfusate}}) \times 100\%$$

Where $C_{\text{perfusate}}$ is the ACV concentration in the perfusate and $C_{\text{dialysate}}$ is the ACV concentration in the dialysate.

The probes were then perfused with normal saline for 40 min to remove residual ACV in the tissue before topical administration of ACV formulations. A dosage of 0.2 g/cm^2 of formulation ACV1, ACV2 or ACV3 was applied to an area of 1.5 × 2.0 cm^2 and then covered with 3M Tegaderm Transparent Dressing (Figure 1). Topical formulations were left in place for 2 h. Microdialysate samples were collected every 30 min up to 4 h.

Statistical analysis

Pharmacokinetic and statistical parameters to determine bioequivalence were calculated with 3P97 software (Chinese Pharmacological Society, Professional Committee of Mathematics). Data were analyzed statistically by one-way analysis of variance and Student's *t*-test using SPSS12.0 software for Windows. A p value < 0.05 was used to determine statistical significance.

Results

In vitro drug release

To investigate both the drug release rate and degree, we performed *in vitro* release of ACV from three different cream formulations. The cumulative amount of ACV (Q) was plotted (Figure 2), and important parameters such

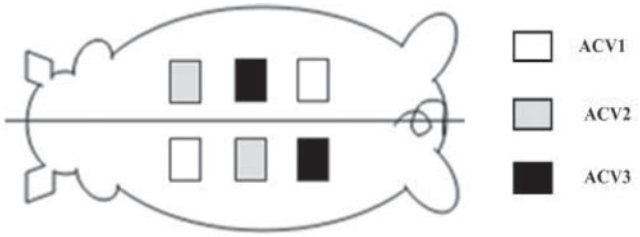

Figure 1. Application sites of three ACV topical formulations on the dorsa of a pig. The pig was anaesthetized by intraperitoneal injection of 30% urethane at a dosage of 3 mL/kg 30 min before each experiment. The application sites for ACV1, ACV2 and ACV3 are indicated. Three microdialysis probes were inserted in the skin on the same side for each experiment.

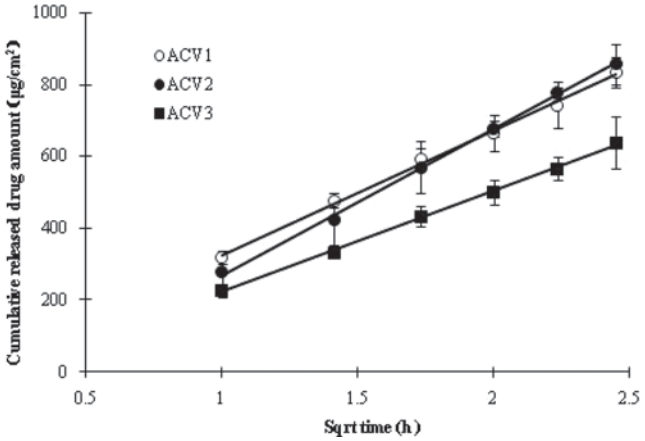

Figure 2. *In vitro* release profiles of ACV (3%) from three topical formulations. ACV from three topical formulations (3%, 0.2 g) was released through a cellulose acetate membrane for up to 6 h. Samples were collected every hour and the amount of ACV released was measured by HPLC. Cumulative amount of ACV was plotted as a function of the square root of time ($t^{1/2}$). Data are presented mean ± SD (n = 4) from two independent experiments.

as cumulative amount, release rate, and correlation coefficient were calculated using linear regression (Table 1).

As shown in Figure 2, accumulative amount of ACV yielded a linear regression (all >0.99) as a function of the square root of time, indicating that ACV was fully dissolved or suspended in these three formulations and that they can be continuously released with a stable release rate. We deduced that these three ACV cream formulations would exhibit a stable release pattern when they are applied *in vivo*, providing a concentration gradient is maintained. The release profiles of these three formulations fit with zero-order kinetics and logarithmic-normal distribution, which is in accordance with the Huguchi distribution[29]. Accumulative release of ACV1 and ACV2 were greater than ACV3 (Table 1), which resulted from different release rates of 346.40, 406.03 and 281.51 μg/cm^{2}h$^{1/2}$ for ACV1, ACV2 and ACV3, respectively. Different release rates observed may be attributed to the specific excipients used in these formulations.

Calibration of the microdialysis probe *in vitro* and *in vivo*

To calculate the probe recovery *in vivo* using the retrodialysis method, three microdialysis probes were calibrated *in vitro* using the ACV standard. To this end, the recovery and delivery efficiency of each probe were measured at three drug concentrations. Our results showed that the *in vitro* recovery and delivery of ACV were independent from drug concentrations (Table 2).

The mean *in vitro* recovery of ACV was 45.71 ± 4.42%, which was comparable to the mean *in vitro* delivery (44.84 ± 1.53%). These results indicated that ACV can consistently passed through the microdialysis probe driven by the concentration gradient, which enabled us to estimate the actual concentration of drugs *in vivo* by retrodialysis as previously reported[22,23]. Furthermore, the recovery and delivery of probe at different flow rates are summarized in Table 3.

Although both the recovery and delivery decreased with increasing flow rates, the recovery and delivery at a certain flow rate remained comparable. In the *in vivo* microdialysis experiment, the mean recovery of microdialysis probes (n = 6) was calibrated by perfusing the ACV standard. The mean recovery of two microdialysis probes used to evaluate ACV1, ACV2 and ACV3 was 35.88 ± 4.12% (12 measurements), 35.10 ± 3.53% (12 measurements), and 34.30 ± 5.16% (12 measurements), respectively. The mean recovery of microdialysis probes (n = 6) was 35.13 ± 3.96% (36 measurements). As expected, the recovery of ACV *in vivo* was lower than that *in vitro* since the diffusion of ACV in skin tissue is much higher than in normal saline *in vitro*. No significant difference in the recovery was observed between each probe *in vivo*. We also documented the probe depth after the skin recovered from microtrauma to avoid the measurement variation (Table 4).

Table 1. Release of ACV from three topical formulations *in vitro*.

Formulation	Cumulative amount (μg/cm^2)	Release rate (μg/cm^{2}h$^{1/2}$)	Correlation coefficient
ACV1	833.22 ± 42.76	346.4	0.998
ACV2	856.96 ± 56.98	406.03	0.999
ACV3	637.98 ± 71.86	281.51	0.999

Three ACV topical formulations (3%, 0.2 g) were separately released through a cellulose acetate membrane for up to 6 h. Samples were collected every hour and the amount of ACV released was measured by HPLC. Data are presented mean ± SD (n = 4) from two independent experiments.

Table 2. Effect of ACV concentration on recovery and delivery of ACV *in vitro*.

Drug conc. (μg/mL)	Recovery (%)	Delivery (%)
0.2	45.85 ± 5.68	45.40 ± 3.13
1.0	45.57 ± 3.17	44.35 ± 2.99
5.0	45.78 ± 4.52	44.96 ± 1.59
Mean	45.71 ± 4.42	44.84 ± 4.42

The *in vitro* recovery and delivery of ACV through the microdialysis probe were studied. The microdialysis probes were perfused with different drug concentrations at a flow rate of 1 μL/min. Data are presented as mean ± SD (n = 3).

Table 3. Effect of flow rate on recovery and delivery of ACV *in vitro*.

Flow rate (μL/min)	Recovery (%)	Delivery (%)
1	45.57 ± 3.17	44.35 ± 2.99
2	32.29 ± 1.62	32.19 ± 1.70
4	19.63 ± 1.23	19.54 ± 1.18

The recovery and delivery of ACV through the microdialysis probe was studied, in which the microdialysis probes were perfused at different flow rates. The drug gradient cross the probe membrane was 1.0 μg/mL. Data are presented mean ± SD (n = 3) from three probes.

Table 4. Probe depth measured for three ACV formulations.

Formulation	Probe depth (mm)
ACV1	1.85 ± 0.37
ACV2	1.88 ± 0.32
ACV3	1.84 ± 0.33

Microdialysis probes were inserted on the dorsa of pigs and the probe depth was measured with the aid of an SA-6000CMT Doppler Scanner. Data are presented mean ± SD (n = 12).

Pharmacokinetic investigation and bioequivalence assessment

To evaluate the bioequivalence of three 3% ACV topical formulations, the pharmacokinetics of ACV in the dermis was investigated. ACV concentrations in dialysate were measured using HPLC and the actual ACV concentrations in the dermis were calculated with the percentage recovery obtained *in vivo* for each probe. All pharmacokinetic parameters were calculated using a one-compartment open model (without lag-time, with weight 1/C) and summarized in Table 5. The results showed that the maximal concentration of ACV in the dermis released topically from ACV1, ACV2 and ACV3 was 1.21 ± 0.53, 0.89 ± 0.48 and 0.82 ± 0.27 μg/mL, respectively (Figure 3).

C_{max} and AUC are commonly used to evaluate bioequivalence for topical formulations according to the FDA guidelines[30]. As we observed in this study, both C_{max}

Table 5. Pharmacokinetic parameters of topically applied ACV creams.

Parameters	ACV1	ACV2	ACV3
C_{max} (μg/mL)	1.21 ± 0.53	0.89 ± 0.48	0.82 ± 0.27
T_{max} (h)	0.33 ± 0.19	0.42 ± 0.25	0.38 ± 0.23
$AUC_{0-\infty}$ (μgh/mL)	1.74 ± 0.65	1.35 ± 0.58	1.37 ± 0.35
$AUC_{0-4\,h}$ (μgh/mL)	1.73 ± 0.66	1.33 ± 0.58	1.35 ± 0.52

Three types of ACV creams (3%) were applied on the dorsa of pigs at a dose of 0.2 g/cm². In DMD, a flow rate of 1 μL/min was used to continuously collect microdialysate, which was analyzed by HPLC. C_{max} indicates maximal concentration; T_{max}, means the time of maximal concentration. AUC (Area Under the Curve) represents the total amount of ACV that was detected in the dermis. Data are presented as the mean ± SD (n = 12).

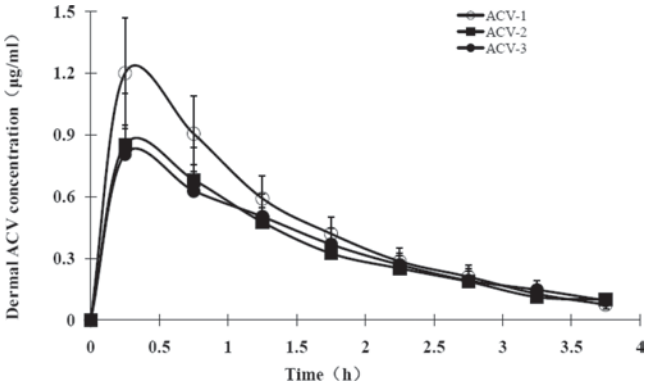

Figure 3. The concentration-time profiles of ACV topically applied on pig skin. Three ACV topical formulations (3%) were applied on the dorsa of pigs at a dosage of 0.2 g/cm² for 2 h. Consecutive microdialysate samples were collected every 30 min in a period of 4 h. Real-time ACV concentration was measured by HPLC. Data are presented mean ± SD (n = 12).

and $AUC_{0-4\,h}$ of ACV in the dermis for ACV1 were significantly higher than those for ACV2 and ACV3 (Table 5), suggesting that ACV1 is not bioequivalent to ACV2 or ACV3. Hence, ACV2 and ACV3 were further evaluated for bioequivalence using log-transformed $AUC_{0-4\,h}$ and C_{max} (Table 6).

Ninety percent confidence interval (CI) for the ratio of log-transformed $AUC_{0-4\,h}$ of ACV2 and ACV3 was 88.2–105.7%, which fell within the acceptance limit (80–125%). Ninety percent CI for the ratio of C_{max} of ACV2 and ACV3 was 87.4–124.4%, which also fell within the acceptance limit (80–125%). However, the power (*i.e.*, the probability of a statistics test to reject the null hypothesis when the null hypothesis is false[31]) obtained for C_{max} was relatively low (67.54%).

Discussion

Bioavailability in the skin is determined not only by the pharmaceutical characteristics of topical formulations, but also by intactness of the stratum corneum. As such, *in vitro* release of topical formulations has limited utility to predict the adsorption of topical drugs *in vivo*. In our study, we found that the rank order of

Table 6. Bioequivalence assessment of ACV2 and ACV3 in pigs.

Parameters	% Ratio (ACV2/ACV3)	90% CI (Lower limit, upper limit)	Power (%)
$AUC_{0-4\,h}$	99.11	88.2, 105.7	92.80
C_{max}	100.48	87.4, 124.4	67.54

Three types of ACV creams (3%) were applied on the dorsa of pigs at a dose of 0.2 g/cm². In DMD, a flow rate of 1 μL/min was used to continuously collect microdialysate, which was analyzed by HPLC. Log-transformed C_{max} and $AUC_{0-4\,h}$ were calculated using 3P97 software (n = 12). The power of a statistical test is the probability that the test will reject the null hypothesis when the null hypothesis is false[31].

the accumulative amount of ACV released *in vitro* from three formulations was ACV1 ≈ ACV2 > ACV3 (Table 1). Although ACV1 and ACV2 had a comparable release rate *in vitro*, we did not observe that ACV2 had a similar diffusion rate with ACV1 *in vivo*. On the other hand, ACV2 showed bioequivalence to ACV3 *in vivo* (with a ranking order of ACV1 > ACV2 ≈ ACV3, Table 5), though the *in vitro* release rate of ACV2 was significantly higher than that of ACV3. Hence, topical delivery of drugs in the dermis cannot be predicted by the *in vitro* drug release because of complicated downstream events such as penetration of stratum corneum and diffusion within the dermis. However, the drug release pattern *in vitro* can serve as a quality control for bioequivalence assessment *in vivo*.

ACV is a water-soluble compound, which makes it more difficult to penetrate into the intercellular lipid-layered stratum corneum than lipid-soluble agents. Previously, several methods have been used to increase the MD probe recovery, by removing the stratum corneum or decreasing the local blood flow rate with the aid of vasoconstriction agents[32,33]. For 5% ACV ointment applied on rabbit skin, Stagni et al. obtained a recovery of ACV of 12.5 ± 3.5% at a flow rate of 3 μL/min for DMD using a microdialysis membrane with a 18-kDa molecular cutoff[22]. For 4% ACV gel applied on rabbit skin, Shukla et al. improved the recovery of ACV by reducing the flow rate to 1 μL/min[33]. Our data also indicate that higher flow rates are associated with lower recovery. At a flow rate of 1 μL/min, the mean recovery of microdialysis probes (n = 6) was 35.13 ± 3.96% (36 measurements). In contrast, no detectable level of ACV was obtained at a flow rate of 5 μL/min after administration of 5% ACV cream on human skin, in which microdialysis probes with a molecular weight cutoff of 2 kDa was used[34]. Thus, reducing the flow rate in DMD sampling can effectively improve the probe recovery for ACV, which can facilitate pharmaceutical analysis.

In this study, we used our previously developed pig model to evaluate bioequivalence of ACV creams. This pig model has several advantages over current animal models for microdialysis based topical drug delivery evaluation. First, the pig has the most similar skin structure and permeability to human skin[35,36]. Although microdialysis studies have been performed on animals such as rat, mouse, rabbit and dog, the pig can mimic to the most extent the actual topical drug delivery in

human skin than other reported species[27]. Second, the dorsal skin of pig has an efficient area of approximately 200–300 cm^2, which is sufficient for simultaneous testing 10 positions. This wide testing area enables replicates of both test and reference formulations in the same subject, thus minimizing the inter-subject variability. Third, the pig model can be used to screen for effective therapeutic agents in the development of topically applied formulations, In this case, performing bioequivalence evaluation in the pig skin model can substantially reduce the safety risk and cost, and provide guidance for future evaluation in clinical trials.

Conclusions

We investigated the bioequivalence of three different ACV formulations applied on pig skin in this study. Due to the similarity between pig skin and human skin, the developed pig skin bioequivalence model can be potentially used to evaluate topical drug candidates prior to clinical evaluation. However, a direct comparison of drug bioequivalence in pigs and in humans needs to be investigated.

Declaration of interest

The authors report no conflicts of interest.

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