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AN APPLICATION OF TRANSDERMAL ANTIVIRAL DELIVERY SYSTEMS TO THE ESTABLISHMENT OF A NOVEL ANIMAL MODEL APPROACH IN THE EFFICACY EVALUATION FOR DERMATOLOGICAL FORMULATIONS

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ABSTRACT

This report described the establishment and the examination of a novel hairless mouse model in the efficacy evaluation for topical antiviral dosage forms with a focus on the relationship between the *in vitro* dermal flux of the antiviral agent and the *in vivo* antiviral efficacy. A unique dose/flux-efficacy relationship in topical antiviral treatment was obtained by applying a series of transdermal acyclovir delivery systems (TADS) for the treatment of cutaneous herpes simplex virus type 1 infected hairless mouse in our earlier study. By konwing the pharmacokinetic parameters of acyclovir in hairless mouse and the flux (J) of a suitable TADS, the skin target site concentration (C*) could be calculated for that patch. With the corresponding C* value, the *in vivo* permeability coefficient ($P_{D,in\ vivo}$) of that patch could be calculated from $P_{D,in\ vivo}$ =J/C*. The difference between this *in vivo* permeability coefficient and the *in vitro* permeability coefficient ($P_{D,in\ vivo}$, obtained from *in vitro* diffusion experiment) was considered due to the blood flow effect in the *in vivo* condition. This $P_{D,in\ vivo}$ can be further applied to calculate the

C* value of any acyclovir topical formulation with flux, J', from $C'^*=J'/P_{D,in\ vivo}$, where the C'* represents the calculated*skin target site concentration* for any acyclovir topical formulation. After knowing different C'* values of different dermatological formulations of acyclovir, the efficacy of each formulation can be estimated from the dose/flux-efficacy relationship. Two formulations with different fluxes were examined under this study. The results showed very good correlation between the *in vitro* acyclovir flux and the *in vivo* antiviral efficacy and the applicability of this model approach was validated.

INTRODUCTION

There are two categories of drug products involved in the percutaneous absorption process. One is dermatological formulations designed to deliver drugs into the skin for managing skin disorders. Since they are designed for topical use, the systemic effects are expected to be as minized as posssible. The other category is transdermal delivery systems designed to deliver drugs through the skin to achieve systermic effects. Because they are designed for systemic use, the topical effects are expected to be as minimized as possible. Initially, transdermal delivery systems were shown to be effective for the treatment of some systemic ailments (1,2). The success of these initial studies encouraged a new era of transdermal delivery systems for the treatment of systemic disorders (3-6). Intensive research and development efforts in the study of the percutaneous absorption process have been stimulated due to the awareness that skin is not very impermeable to lipophilic drugs and is a potential portal of entry for drugs into the body (7,8).

Due to the prosperous study on percutaneous absorption, scientists have started to pay attention to the problems and issues in the development and optimization of dermatological products (9-11). One of these issues is to establish an accepted non-clinical model or approach to predict the bioavailability and bioequivalence of dermatological formulations. Since the barrier properties of the skin reside primarily in the stratum corneum (12-14), it is very reasonable to do *in vitro* determination of rate of absorption directly below the skin membrane. Because the absorption process is a passive diffusion through a primary barrier of nonliving tissue, it is not surprising that *in vitro* and *in vivo* absorption comparisons have generally been favorable. Due to the difficulty in doing *in vivo* human studies, most of the comparisons thus far have been made with animal skin. However, there are many factors (7, 15-17) which can influence the delivery of

drugs through the skin (such as: skin hydration (7, 8, 14, 18); temperature effects (19); barrier properties as function of site (20), age (21, 22), race (23) , conditioning of skin (24-26), health of skin (27), animal species (28); blood flow and dermal clearance (8); occlusion's effect (29); biotransformations (30); etc...). Therefore, no one has been able to establish any guidelines with regard to the assessment and use of laboratory models in the predicting and optimizing the clinical efficacy of topical formulations.

The purpose of this presentation is trying to introduce our efforts in developing an *in vivo* animal model and demonstrating a unique example of a dermal flux-response relationship in the treatment of cutaneous herpes simplex virus type 1 (HSV-1) infected hairless mice using (trans)dermal acyclovir delivery systems (31). By using that well developed hairless mouse model in our laboratories, we are able to establish as well as examine carefully a novel method to assess bioavailability and to predict topical efficacy of antiviral agents using finite dose acyclovir in the treatment of cutaneous HSV-1 infections in the hairless mouse model. This novel method is based on the measurement of the free drug concentration (C^*), or the thermodynamic activity of drug at the skin target site (32).

DEVELOPMENT OF TRANSDERMAL ACYCLOVIR DELIVERY SYSTEMS

Acyclovir was chosen as the model drug due to its high antiviral activity and selectivity for herpes viruses (33,34). The preparation of acyclovir free acid was described in detail in our previous publications (31).

Fabrication of the (Trans)dermal Delivery System

Hydrogel membranes were used for controlling ACV fluxes through the azone-pretreated hairless mouse skin as in our previous study (31). Without azone pretreatment, the stratum corneum would be the rate-limiting membrane not the hydrogel membrane. The preparation and characterization of these hydrogel membranes have been previously (31, 35) described in detail. Briefly, the membranes were prepared by homopolymerization of 2-hydroxyethyl-methacrylate (HEMA) or copolymerization of HEMA with styrene (Sty) or N-vinylpyrrolidone (VP) using ethylene glycol dimethacrylate (EGDMA) as crosslinker and 2,2'-azobisisobutyronitrile (AIBN) as initiator. The polymerization were carried out in bulk in polyethylene molds. By varying the chemical composition, different

hydration values of the membranes were obtained. It was shown that the permeability of these hydrogel membranes to different solutes increased with their hydration ability over a wide range. The drug delivery rate of the dermal patches could be regulated using these hydrogel membranes. All the starting materials for the preparation of these hydrogel membranes were obtained from Polyscience, Warrington, PA. Monomers were purified by distillation under reduced pressure prior to use, while EGDMA and AIBN were used as received.

The fabrication of the (trans)dermal acyclovir delivery systems (TADS) has been well developed in our laboratories. Basically, the patch consisted a backing film, a Silastic O-ring a feeding tube and a rate controlling membrane. The drug reservoir in each TADS was loaded with a suspension of ACV sufficient for constant rate release over the experimental period. The tube was then heat sealed. The drug, ACV, was suspened in 0.2% aqueous carbopol gel (pH 5.0). Carbopol® 934P (B. F. Goodrich Co., Cleveland, OH) was used as received.

Drug Delivery Rate of the Dermal Patches

Figure 1 showed typical *in vitro* drug release profiles of dermal patches with four different compositions of controlling membranes. The results demonstrated that the patches provided constant zero-order release rates.

VIRUS AND ANIMAL MODEL

Virus

Samples of the same batch herpes simplex virus type 1, strain E-377, with a titer of 1.35×10^8 plaque-forming units (PFU)/ml were used throughout this study. They were stored in aliquots at -70 °C until used. The preparation and assay of the virus have been previously described and performed (36) in Dr. E. R. Kern's laboratory (Department of Pediatrics, University of Alabama at Birmingham).

Animal Model

Female hairless mice (strain SKH/HR1), 6-7 weeks old with an average body weight of 23-24 g were used throughout this study. The age of the mice was critical in these studies, since mice younger than 6-7 weeks suffered strong skin irritation after the azone pretreatment and with mice older than 6-7 weeks the

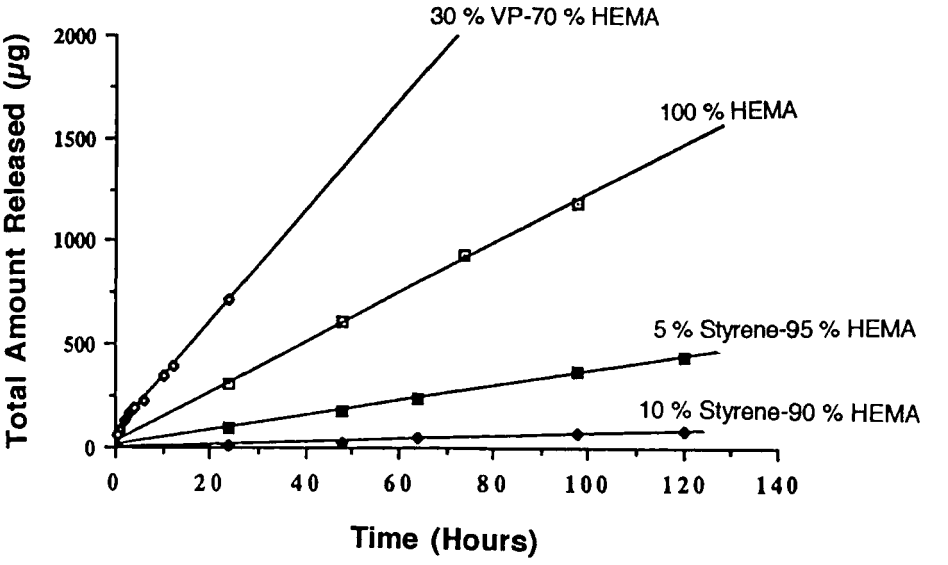

FIGURE 1

Release Profiles of ACV from Dermal Patches with Different Compositions of Controlling Membranes. Membrane thickness were about 1 mm.

reproducibility of the virus infection was found to decline (unpublished data). Hairless mouse were chosen because it does not have hair and is easier to do penetration experiments and to apply topical formulations with this skin. The properties of transport and HSV-1 infection of hairless mice skin have been well characterized in our laboratory (30). As described in our previous paper (31), the infection induced by the HSV-1 inoculation generated skin lesion. This lesion appeared at the site of inoculation as early as the third day after inoculation and progressed in the form of a 4-5 mm wide band first towards the back (dorsum) and then towards the abdomen of the mice. A lesion generally was fully developed 5-6 days after inoculation and formed a continuous band extending from the spinal area to the middle of the abdomen. All of the hairless mice died from encephalitis by the seventh or eighth day after inoculation. Although very little properties of physiology of these mice were known, they have been considered to be immunologically hyporesponsive (37) or functionally immunologically deficient (38). Female hairless mice (strain SKH/HR-1), 5-6 weeks old with body weight 20 ± 2

g, were purchased from Temple University, Philadelphia, PA and used in this study.

Categorization of Lesion Developments and Measurement of Antiviral Efficacy

The antiviral efficacy of both the topical and the systemic effects, was evaluated based on the extent of lesion development in the skin area adjacent to and underneath the patches which can be classified into five categories, namely, "Not Reach (NR)", "Stop (St)", "Jump (J)", "Through (Th)", and "Miss (M)". These categories have been clearly illustrated and described in detail in our previous work (31). Briefly, NR (not reach) represents that the lesions started at the site of the virus inoculation and developed only to a limited extent without reaching the dermal patch area. In this case, the drug concentrations in the systemic circulation reached the levels capable of inhibiting virus replication in skin. Thus, NR was interpreted as a measure of systemic effectiveness. A different thinking for "NR" is that the ACV concentration at the end of lesions (0.5 to 1 mm distant from the edge of the patch in most of the cases) would be very small and not effective by topical diffusion , because the parallel flux (x- or y-direction) was 500- to 1000-fold less than the perpendicular flux (z- direction). Th (through) represents that the lesions formed a continuous band which grew across (through) the skin area covered by the dermal patch. In this case, the drug concentrations achieved by systemic or topical delivery were not high enough to show an antiviral activity. St (stop) represents that the lesions reached the edge of the dermal patch, but did not develop in the skin area covered by the patch. J (jump) is the case that lesions developed in a band form on both sides of the dermal patch but no lesions were visible in the area covered by the patch. In these two cases, the drug concentrations that could inhibit virus replication were obtained only in the skin area covered by the dermal patches, i.e., in the area of topical drug delivery. M (miss) is the case that the lesions grew irregularly, not forming a straight band and therefore missing the dermal patch area. These lesion categories, except for "M", are indications of the effectiveness of ACV delivered systemically and/or topically in curtailing virus replication and virus-induced skin lesion. Therefore, the above category designations of lesion development were interpreted in terms of antiviral drug effectiveness as follows:

$$\text{topical efficacy (\%)} = \frac{N_{St} + N_J + N_{NR}}{N_{St} + N_J + N_{Th} + N_{NR}} \times 100\% \qquad [1]$$

$$\text{systemic efficacy (\%)} = \frac{N_{NR}}{N_{St} + N_J + N_{Th} + N_M + N_{N\cdot R}} \times 100\% \quad [2]$$

where N_{St}, N_J, N_{NR}, N_{Th}, and N_M are the numbers of animals corresponding to each of the five lesion categories in each experimental group.

IN VIVO ANTIVIRAL EFFICACIES

Table 1 presents the results of antiviral efficacies calculated based on the numbers of animals corresponding to each of the five lesion categories in each experimental group from *in vivo* studies conducted with different time delayed ACV treatments. Lesion "scoring" was performed on day 5 postinoculation; this choice is based on our previous (31) experience that five days represented an optimum when a fully developed lesion (25 mm or longer) could be identified in all the control animals while the mortality rates of the animals were still low. Included in Table 1 for comparison are the results of placebo groups in each experimental run. As can be seen, all animals in the placebo groups developed a "Th" lesion category five days after virus inoculation.

To clearly illustrate the relationship between the antiviral efficacy and the amount of ACV released and to better appreciate the differences and similarities in the antiviral effectiveness between the different time delayed ACV treatments, both the topical and the systemic efficacy are plotted in Figure 2 as a function of the experimental flux.

It is clear from Figure 2 (each data point represents the calculated antiviral efficacy based on a group of 8 - 10 animals) that the results importantly showed that there is a strong relationship between ACV flux and topical efficacy. At low ACV flux from 10 to 100 $\mu g/cm^2$-day, the topical efficacy increases with increasing flux; when the ACV flux is 100 $\mu g/cm^2$-day or greater, the topical efficacy reaches a maximum of 100%. However, when the ACV treatment was delayed for three days, the topical efficacy appeared to be much worse than that of zero-day delayed treatments. This outcome is reasonable considering that we have observed in several cases in the control animals fully developed skin lesions (25 mm or longer) by day 3.5 postinoculation and ACV therapy initiated on day 3 could likely be too late to curtail the further development of a skin lesion, especially when the ACV flux is low.

TABLE 1

Antiviral Efficacies in *In Vivo* Experiments with Delayed ACV Treatments.

Expt group	Delayed treatment	ACV flux, theo ($\mu g/cm^2/day$)	ACV flux, exp ($\mu g/cm^2/day$)	n	No. of animals with lesion category of					Topical efficacy (%)	Systemic efficacy (%)
					St	J	Th	NR	M		
1-0		Placebo	Placebo	9	0	0	9	0	0	0	0
1-1	1 day	231	119	10	6	2	0	2	0	100	20
1-2		107	70	8	3	3	1	1	0	88	13
2-0		Placebo	Placebo	6	0	0	6	0	0	0	0
2-1	2 days	94	50	9	0	5	2	2	0	78	22
2-2		187	100	9	1	6	0	2	0	100	22
3-0		Placebo	Placebo	10	0	0	10	0	0	0	0
3-1	2 days	39	24	10	0	3	6	1	0	40	10
3-2		644	421	10	0	1	0	9	0	100	90
4-0		Placebo	Placebo	10	0	0	10	0	0	0	0
4-1	3 days	69	64	10	0	1	9	0	0	10	0
4-2		334	331	10	1	2	1	4	2	88	40

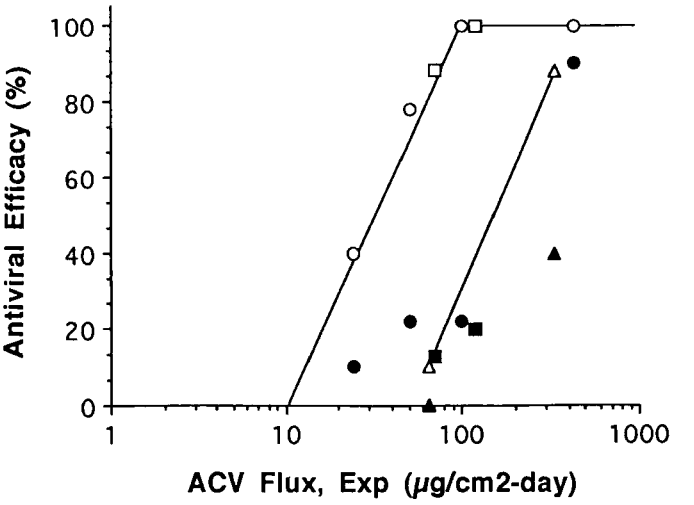

FIGURE 2

The topical (open symbols) and the systemic (closed symbols) antiviral efficacy presented as a function of the experimental ACV flux in *in vivo* experiments with one- (□, ■), two- (○, ●), and three-day (△, ▲) delayed ACV treatments. Each data point represents the calculated antiviral efficacy based on a group of 8-10 animals.

The systemic efficacy results support the view (see Fig. 2) that higher fluxes are required to treat cutaneous herpes infections via the systemic route in hairless mice than for treatment via the direct topical route. These data are consistent with the thinking that in topical therapy as opposed to systemic therapy, there would be an ACV concentration gradient from the epidermis towards the dermis at the site of lesion development; therefore, lower ACV fluxes would suffice to maintain a steady-state ACV concentration in the epidermis that would be sufficiently high to inhibit virus replication *in vivo*. In systemic therapy, higher fluxes would generally be needed to achieve the same steady-state epidermis ACV levels as high steady-state systemic blood levels would be required to adequately equilibrate the target epidermis from the systemic blood compartment. After extended examination of this antiviral efficacies with the TADS applied for different time periods, we are very comfortable about this flux-efficacy relationship outcome.

STRATEGY OF STUDIES

So far we have established a way to make TADS and obtained a good flux-efficacy relationship for these patches, it is time to apply this concept to topical formulations. From the physicochemical point of view, the temporal pattern of the free drug concentration (C*), or the thermodynamic activity of a drug, at the skin target site would be expected to be a measure of bioavailability (1, 37, 38) for topical formulations. Establishing bioequivalence is then to demonstrate in the generic version of topical drug products a comparable pattern of C* changes with time as the innovator's formulation under the same dosing regimen. Based on the above interpretation, a correlation is expected to exist between the temporal pattern of C* and the clinical effectiveness of a dermatological formulation and the issue of predicting clinical effectiveness then may become a matter of determining the time course of C*. If the C* versus time profile lies within the therapeutic window for the drug, the formulation under investigation may be ruled as effective. Furthermore, if the temporal patterns of C* for two or more drug products are essentially superimposable, then it is proposed that the different formulations are bioequivalent. This bioequivalent statement should be true for an acyclovir formulation administered systemically and for another formulation administered topically. Therefore, the following presentation will be focused on (a) the assessment of C* from TADS (b) the calculation of $P_{D,in\ vivo}$ (c) the preparation of a topical formulation and the calculation of its flux and its skin target site concentration and (d) the examination of the correlation between the *in vitro* flux and the *in vivo* efficacy results.

THE DETERMINATION OF C^*_{50} (THE C* FOR 50 % ANTIVIRAL EFFICACY)

In a recent review (9), it has been recommended that biopsy, skin section and assay for drug in the various tissue layers should be used to determine bioavailability for antivirals. However, for skin samples, such as those obtained from hairless mice, which cannot be easily sectioned without destroying the integrity of skin layers, the assessment of C* presents an unprecedented challenge. In this paper, we introduce a novel approach which estimates the systemic C^*_{50} from the steady-state plasma free drug concentration (41).

The following procedure and data analysis provide a value for C^*_{50}. Three transdermal acyclovir patches which produced an ACV flux responsible for a 50 %

systemic efficacy (see Figure 2) were applied to the hairless mouse skin for a fixed time period in an area pretreated with azone and the ACV level in the systemic circulation was determined daily to establish the steady-state blood concentration. In practice, the experimental steady-state blood level was in good agreement with the theoretical prediction based on a two-compartment model analysis:

$$C = \frac{JS}{V_c k_{el}}\left(1 + \frac{\beta - k_{el}}{\alpha - \beta}e^{-\alpha t} + \frac{k_{el} - \alpha}{\alpha - \beta}e^{-\beta t}\right) \quad [3]$$

where C is the blood concentration, J is the drug flux, S is the diffusional surface area of the TADS, V_c is the apparent volume of distribution of the central compartment, k_{el} is the elimination rate constant, α and β are the disposition rate constants, and t is the time. All pharmacokinetic parameters involved in the calculation were obtained from an i.v. bolus injection experiment. In our current example, the prevailing ACV blood concentration at steady-state was found to be 0.25 ± 0.11 μg/ml.

It is believed that only the unbound fraction of drugs in the plasma compartment is equilibrated with C* at the epidermal target site. In a separate experiment (41) which studied the *in vitro* partitioning of ACV between red blood cells and plasma, the ratio of ACV concentration in the plasma to that in the whole blood at equilibrium was estimated to be 1.6. This implied that the concentration of ACV in the red blood cells and in the plasma was about the same. Therefore, the steady-state plasma ACV concentration was determined as 0.25 ± 0.11 μg/ml. Also, it has been reported (42) that there is practically no binding of ACV to plasma proteins in mice at low plasma ACV concentrations (2.7 μg/ml or less) based on the results of *in vitro* binding studies. Thus, the plasma free ACV concentration at steady-state would be equal to 0.25 μg/ml. This steady-state free ACV concentration in the plasma was equated to the C* in the aqueous compartment of the epidermal target site; an assumption which we believe is reasonably good for the following reasons: (a) there is little or no ACV metabolism in the skin (43), and (b) distribution of the free ACV between the plasma and the aqueous compartment of the epidermal target site is believed to take place very quickly. For ACV and other small drugs with a molecular weight in the range of 100-500, the effective diffusion coefficient (D) for this class of drugs in the dermis of hairless mice (30)

would be generally around 1 x 10^{-6} cm^2/s and therefore, C* in the aqueous compartment of the epidermal target site could reach its steady-state value in the order of minutes depending on the choice of dermis thicknesses (h) in the following equation:

$$t = \frac{h^2}{6D} \qquad [4]$$

It is worth of mentioning that the systemic C^*_{50} value (0.25 μg/ml) determined in our study either falls within the range of (44-50), or is within an order of magnitude greater (51-54) or smaller (55, 56) than the literature values of ID_{50}. Here, ID_{50} is the ACV concentration required *in vitro* to inhibit HSV-1 induced cytopathogenicity or viral plaques by 50 % in Vero cell cultures.

The following analysis examines the relationship of C^*_{50} determined above (from the systemic efficacy data) to the topical efficacy results. For a drug with no appreciable skin metabolism, such as ACV, we may write (32):

$$J_{50} = P_D(C^*_{50} - C_b) \qquad [5]$$

where J_{50} is the flux for 50 % topical efficacy, P_D is the permeability coefficient for the drug in dermis and C_b is the drug concentration in the circulating blood and is assumed to be negligible compared to C^*_{50}.

Rearrange equation [5], we have:

$$C^*_{50} = \frac{J_{50}}{P_D} \qquad [6]$$

Now, we find an important inconsistency between the C^*_{50} obtained using equation [6] with an *in vitro* P_D value and that obtained from the systemic efficacy studies (above). From Fig. 2, J_{50} is found to be 30 ± 2.3 $\mu g/cm^2$-day. For P_D, a value of (6.8 ± 0.5) x 10^{-5} cm/s was obtained from *in vitro* percutaneous absorption studies of ACV with heat-separated dermis (A. Gonsho, unpublished results). Substitution of these two values into equation [6] gives a topical C^*_{50} of

5.1 ± 0.8 μg/ml. This value is approximately 20 times greater than that obtained systemically (0.25 ± 0.11 μg/ml).

From clinical pharmacology considerations, the C^*_{50} achieved topically would be expected to be the same as the C^*_{50} obtained systemically. Since the systemic C^*_{50} is estimated directly from steady-state blood level analysis, the 20-fold difference in the C^*_{50} values is most easily attributable to an error in the topical C^*_{50} calculation. In this regard, it has been shown (8, 57-62) in other studies that the *in vitro* drug distribution profiles in the skin at a given time do not agree with the *in vivo* results and that the dermal drug concentrations are generally higher in the former cases due to the absence of a functioning blood transport system. This is similar to the condition where the dermis thickness has effectively become much thinner *in vivo* compared to *in vitro*. Thus, the P_D value of 6.8 x 10^{-5} cm/s is consistent with a full-thickness dermis of 250 μm, and this value is inappropriate for *in vivo* analysis. Only a much thinner dermis thickness (i.e., 1/20 of the anatomical thickness, or ~ 13 μm) and a correspondingly larger P_D (the *in vivo* value) would give agreement between C^*_{50} obtained with equation [6] and the C^*_{50} obtained from the systemic studies.

It should be emphasized that the *in vivo* dermis thickness of 13 μm determined from our model analysis using the hairless mouse skin is anatomically reasonable. As reported by Hammersen (63), after histological studies of human skin, he found that the shortest distance between the capillary loop and the epidermal basal layer is estimated to be 3-4 mm under the microscope and this translates into a separation of 17-23 μm in real skin samples. In addition, it has been reported in one paper (64) that a rich bed of capillaries is encountered by the drugs delivered topically 20 μm or so into the dermis field in the human skin. Still in another report (65), the microcirculation has been estimated to occur in the human skin from a depth of 26-76 μm into the dermis.

IN VITRO ACV FLUX DETERMINATIONS AND *IN VIVO* C* ASSESSMENTS

During the early stage of experimental design, we were confronted with two major practical problems. The first problem encountered was how to conduct

meaningful *in vitro* 3-day or a 4-day flux studies when it is well-documented (67, 68) that hairless mouse skin is not stable, *in vitro*, beyond around 24 hours in a diffusional cell experiment. The way we overcome this problem was to implement a combined *in vivo-in vitro* experiment to obtain relevant *in vitro* fluxes. Specifically, topical ACV formulations are applied onto live animals following the same protocol as in the*in vivo* efficacy studies, then at the predetermined point in time the animals are sacrificed and the ACV-treated skin excised, and the Franz cell *in vitro* flux experiments run for 24 hours. For example, to obtain the first day flux results, skin samples are excised from freshly killed animal to determine the *in vitro* fluxes for 24 hours; to obtain the day 2 flux, the*in vivo* experiment is run for one day before skin samples are excised to determine the *in vitro* fluxes on day 2; to obtain the day 3 flux, the*in vivo* experiment is conducted over the first two days and the *in vitro* fluxes determined on day 3; and so on. The complete experimental design is presented in Table 2.

The second major problem encountered was how to protect the topically applied formulation from being licked off, scratched off, and/or rubbed off once applied onto the hairless mouse skin. We tried different designs of restrainers, without success. Finally, a Velcro jacket was made which covered the entire trunk of the hairless mouse except for the part (1" x 1" in dimension) at the left back area, which was designated for the application of formulations. A Velcro cover for protecting the drug treated part of the animal but allowing for good ventilation was also made. The details of this Velcro protection jacket are illustrated in Fig. 3. It has turned out that this Velcro protection jacket serves its purpose quite well and the mouse is able to lead a normal life pattern wearing this jacket.

Although the commercially available Zovirax 5% ointment provided us with a convenient sample to test with our hairless mouse model, it was considered desirable to examine one or two more topical formulations so that our claim on the applicability of this model approach would be much more confirm. However, the search for a topical formulation which displayed a desired ACV flux profile was no easy task, especially as the desired profile falls within a very narrow flux range (between 10 and 30 $\mu g/cm^2$-day). Using trial and error, we were fortunate however to come up with a topical formulation (3% ACV suspension) whose ACV flux profile closely matched what we had in mind. The preparation method of this topical formulation was described in detail by Lee et al. (66).

TABLE 2

The Combined *In Vivo-In Vitro* Experimental Design for Determining the *In Vitro* ACV Flux Across the Hairless Mouse Skin Using Finite Dose Formulations [a].

Results Obtained \ Day	Day 1	Day 2	Day 3	Day 4	Day 5
First Day Flux	_____b				
Second Day Flux	* *c	_____b			
Third Day Flux	* *c	* *c	_____b		
Fourth Day Flux	* *c	* *c	* *c	_____b	
Fifth Day Flux	* *c	* *c	* *c	* *c	_____b

a. Applicable to both Zovirax 5% ointment and the self-prepared 3% ACV suspension.
b. *In vitro* flux determinations conducted with freshly prepared skin samples using a Franz cell at 37°C for 24 hours.
c. The *in vivo* part of a combined *in vivo-in vitro* experiment. Every 12 hours, the residual ACV formulation on the skin was removed and a fresh dose reapplied. Each animal wore a Velcro protection jacket during this period and was individually housed.

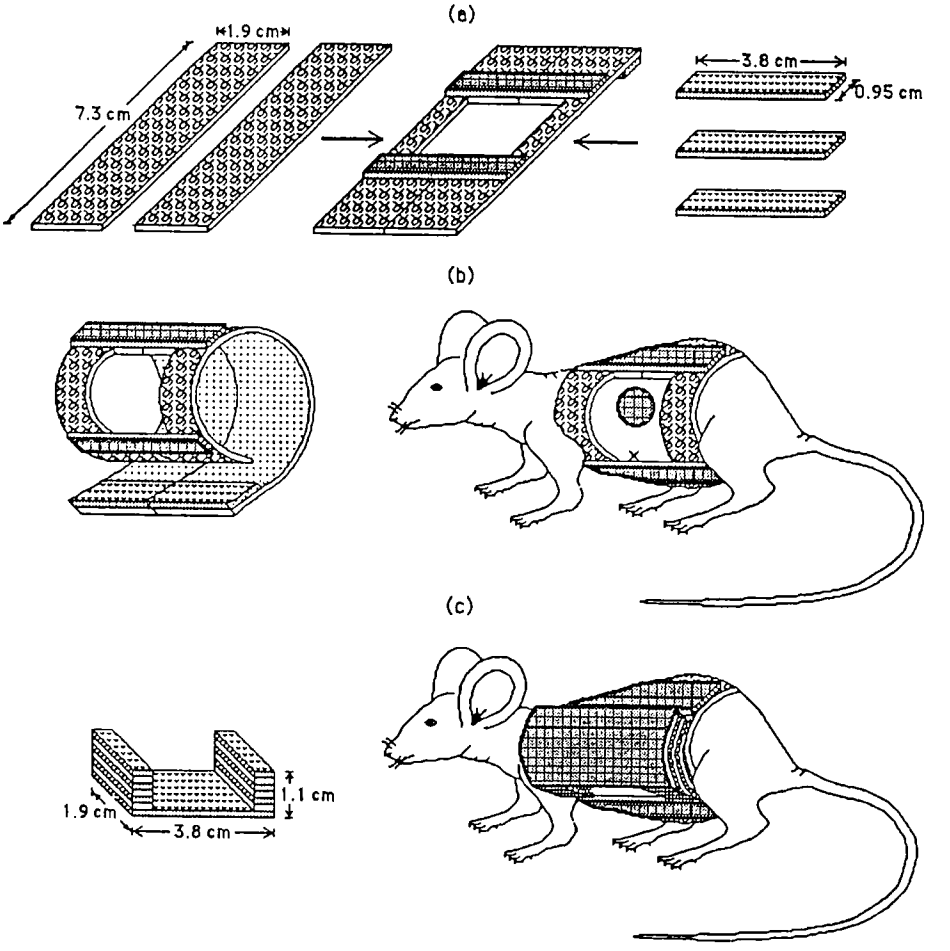

FIGURE 3

A schematic illustration of a Velcro protection jacket which was fabricated by (a) Fastening two pieces of the hoop-and-loop Velcro together with a 1" x 1" opening in the center to expose the virus inoculation site and the skin area (1 cm^2) for application of the finite dose formulation. (b) The finished jacket was wrapped around the trunk of a hairless mouse. (c) Once the formulation was applied, another piece of Velcro cover was mounted over the opening for protection but leaving plenty of room for ventilation.

During the *in vivo* part of the combined *in vivo-in vitro* experiment for Zovirax ointment, a circular skin site with an area equivalent to the diffusional area (1 cm^2) of the Franz cell on the left back area of a hairless mouse was treated with finite dose topical formulations every twelve hours. For Zovirax 5% ointment, the dose given was 3 mg and was applied onto the skin with inunction using a small glass rod, while for the self-prepared 3% ACV suspension, the dose given was 2 μl and was slowly added onto the skin using a micropipette; this maximized the chance for an even distribution of the drug suspension over the skin surface. Each hairless mouse wore a Velcro protection jacket during the entire *in vivo* experimental period and was individually housed. At the end of each dosing interval, the small piece of Velcro cover over the formulation application site was temporarily detached and the residual formulation on the skin was wiped clean with a cotton-tipped applicator prewetted with saline prior to the application of the next dose.

To begin the *in vitro* part of the combined *in vivo-in vitro* experiment, the Velcro protection jacket was removed from each hairless mouse and the remaining drug formulation on the skin was wiped clean. The hairless mouse was then sacrificed by cervical dislocation and the formulation-pretreated skin site with its surrounding tissue was excised. The procedures were described in detail by Lee et al. (66). The collected samples were analyzed for their ACV contents using HPLC. The amount of ACV permeated through the hairless mouse skin was plotted as a function of time and the instantaneous ACV flux (J') was estimated from the slope of the line connecting the two consecutive time points (differential method). The C'* for the topical formulation was calculated using the following equation :

$$C'^{*} = \frac{J'}{P_{D,\text{in vivo}}} \qquad [7]$$

where $P_D = (1.4 \pm 0.72) \times 10^{-3}$ cm/sec and C'* is used for the skin target site concentration of a topical formulation in order not to muddle up C* calculated from TADS.

IN VIVO ANTIVIRAL EFFICACY STUDIES

The *in vivo* antiviral efficacy studies with finite dose topical ACV formulations were conducted using ten animals in each group. The hairless mice were inoculated with HSV-1 following the procedures described in our previous work (31). For the treatment, the finite dose formulations were applied onto a circular skin site (1 cm^2 in area) on the left back area about 1 cm distance dorsal to the virus inoculation site and in the predicted path of lesion development and a one-day delayed four-day treatment protocol (69) was used (i.e., the treatment would start one day after the virus inoculation and continue for four consecutive days). The dosing interval, the doses given, and the ways to apply the doses and to remove the residual formulations were exactly the same as those described above in the *in vivo* part of the combined *in vivo-in vitro* experiment. Each hairless mouse wore a Velcro protection jacket during the treatment period and was individually housed. Five days postinoculation, the Velcro protection jacket was removed and the lesion development was scored for each mouse and used to calculate the antiviral efficacy of ACV as described by Lee et al. (69).

For control, a placebo group was always run along with each experimental group. For the present study, the placebo group was treated with a (trans)dermal patch containing aqueous Carbopol (B. F. Goodrich Co., Cleveland, OH) gel but without ACV as in our earlier studies (31,69).

Also run for control purpose in each experiment was the group(s) treated with a (trans)dermal patch containing an ACV suspension to ensure that the results were reproducible under different circumstances. This experiment also permitted a direct comparison of a current study with previous baseline efficacy studies using the (trans)dermal patch delivery system. The treatment with (trans)dermal patches used a two-day delayed three-day treatment protocol (69).

In Vitro ACV Flux Determinations and *In Vivo* C'^{*} Assessments Using Finite Dose Zovirax 5% Ointment

Figure 4 shows the *in vitro* ACV release profiles of (3 mg) Zovirax 5% ointment in a combined *in vivo-in vitro* experiment. Unlike the *in vivo* part of the combined *in vivo-in vitro* experiment where the finite doses were given every 12 hours, the *in vitro* flux determinations were run for 24 hours. This is because (a) it was found difficult to remove the residual formulation and to apply a new dose

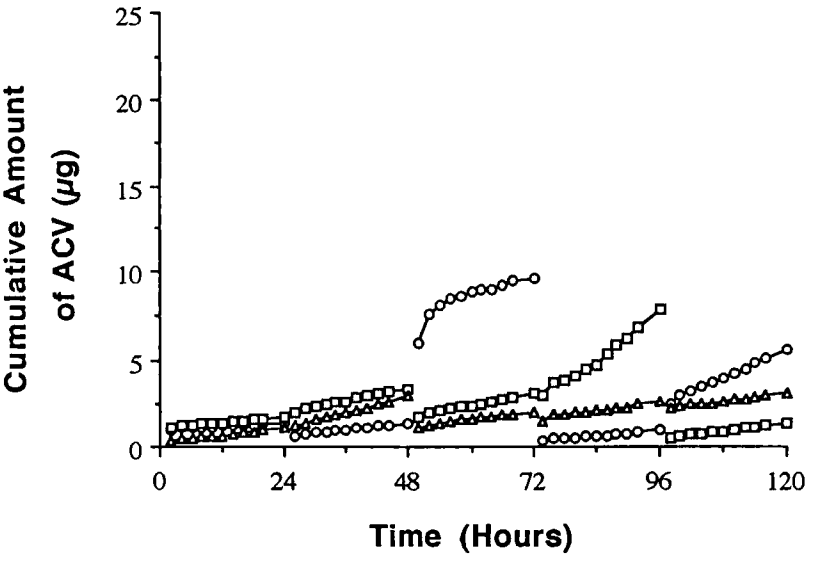

FIGURE 4

The accumulated amounts of ACV delivered topically across the hairless mouse skin into the receiver chamber of a Franz cell using a 3 mg finite dose Zovirax 5% ointment in a combined *in vivo-in vitro* experiment. The experiment was run in triplicate.

onto the isolated skin samples which became very delicate through hydration for 12 hours; and (b) our preliminary studies had shown that the ACV fluxes (the slopes) usually remained relatively constant for 24 hours during the *in vitro* Franz cell experiments.

It is clear from Fig. 4 that except for one skin sample from each of the third, the fourth and the fifth day, the *in vitro* ACV flux results (the slopes) were quite reproducible and remained relatively similar over the five-day experimental period. This outcome was most satisfying for our model study as it minimized the variations of C'^{*} during the treatment period and helped in the more exact interpreting of the results.

Figure 5 shows the temporal pattern of C'^{*} assessed according to Eqn. [7] using the instantaneous ACV fluxes estimated from the slopes of the lines connecting the two consecutive time points in Figure 4. As can be inferred from the *in vitro* ACV fluxes (the slopes), the C'^{*} also remained relatively constant

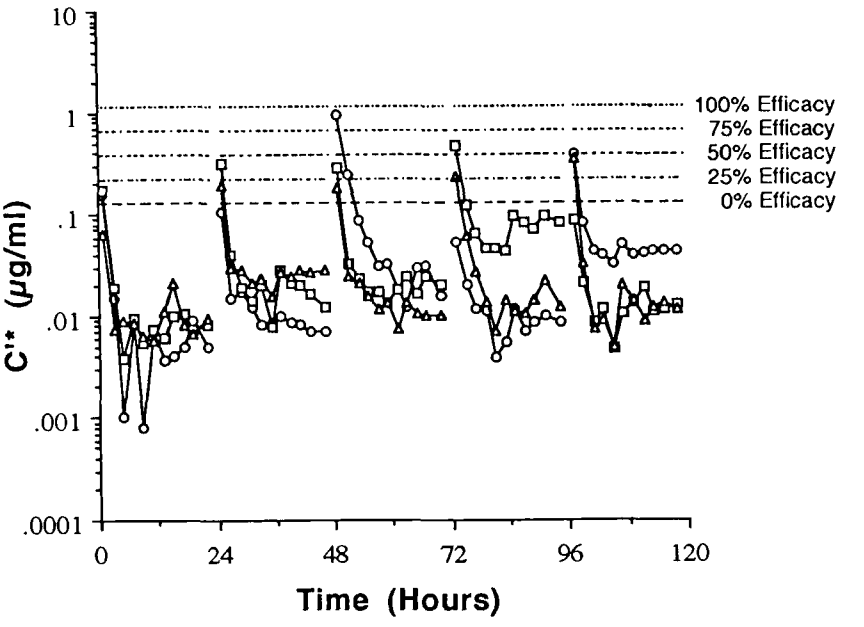

FIGURE 5

The temporal pattern of C'* assessed for Zovirax 5% ointment according to Eqn. [7] using the instant ACV fluxes estimated from the slopes of the lines connecting the two consecutive time points in Fig. 4. The broken lines represent the C* values corresponding to the different antiviral efficacy calculated based on our previous results (69).

throughout the five-day period. It is important to note here that the C'* values stayed far belowthat corresponding to a 0% topical efficacy; consequently, these results predict poor efficacy for Zovirax 5% ointment in this model.

In Vivo Antiviral Efficacy Study Using Finite Dose Zovirax 5% Ointment and Correlation between the *In Vitro* Flux and the *In Vivo* Efficacy Results

The results of the *in vivo* antiviral efficacy study obtained from the experimental group treated with finite dose (3 mg) Zovirax 5% ointment and from the placebo and the two control groups treated with (trans)dermal patches are presented in Table 3. The five lesion categories used here were exactly the same as described previously, namely, the lesion development went through (Th), stopped (St) at the edge of, jumped (J) over, did not reach (NR), and totally missed (M) the

TABLE 3

Results of the In Vivo Antiviral Efficacy Study Using Finite Dose Zovirax 5% Ointment

Group	Lesion Category for Mouse No.										Antiviral Efficacy	
	1	2	3	4	5	6	7	8	9	10	Topical	Systemic
Placebo [a]	Th	Th	Th	Th	Th	Th	Th	Th	Th	Th	0 %	0 %
Control I [b]	St	St	St	St	St	NR	St	St	M	St	100 %	10 %
Control II [c]	St	Th	Th	NR	St	Th	J	Th	Th	Th	40 %	10 %
Experimental [d]	Th	Th	Th	Th	Th	Th	Th	Th	Th	--[e]	0 %	0 %

a. Treated with a (trans)dermal patch containing aqueous Carbopol gel but without ACV employing a two-day delayed three-day treatment protocol.
b. Treated with a (trans)dermal patch containing an ACV suspension employing a two-day delayed three-day treatment protocol. The (trans)dermal patch used a rate-controlling membrane made up of 100% HEMA with 1 mm thickness.
c. Same as b except that the (trans)dermal patch used a rate-controlling membrane made up of 90% HEMA and 10% styrene with a thickness of 0.5 mm.
d. Treated with finite dose (3 mg) Zovirax 5% ointment every 12 hours employing a one-day delayed four-day treatment protocol.
e. The mouse died prior to the lesion scoring.

designated skin site for treatment with the (trans)dermal patch or with the finite dose formulation.

The placebo group treated with a (trans)dermal patch containing no ACV showed a 100% infection rate, i.e., all ten animals in this group displayed a "Th" lesion category, indicating that the batch of virus used was viable and there should be no doubt about our technique for virus inoculation.

The two control groups were treated with (trans)dermal patches containing ACV employing a two-day delayed three-day treated protocol. For control group I, the (trans)dermal patch used a rate-controlling membrane made up of 100 % 2-hydroxyethylmethacrylate (HEMA) with 1 mm thickness. The ACV flux for this (trans)dermal patch was 416 $\mu g/cm^2$-day determined *in vitro* using the method described previously and was 325 $\mu g/cm^2$-day estimated *in vivo* using the method of extraction (69). The possible reasons for the *in vivo* flux being smaller than the *in vitro* prediction have been discussed in detail by Lee et al. (69). The control group I demonstrated a 100% topical efficacy which was consistent with our previous results and a 10% systemic efficacy which was a little lower than our previous results (69). For control group II, a rate-controlling membrane made up of 90% HEMA and 10% styrene with a thickness of 0.5 mm was used in the preparation of (trans)dermal patches. The results in Table 3 showed that this second control group had a topical efficacy of 40% and a systemic efficacy of 10% and both were in general agreement with our previous results (69).

For the experimental group, the animals were treated with finite dose (3 mg) Zovirax 5% ointment every 12 hours using a one-day delayed four-day treatment protocol. This treatment protocol was considered a reasonable choice; we had previously shown with the (trans)dermal patch that zero-day, one-day, and two-day delay treatment protocols gave essentially the same results (69). As can be seen from the bottom row of Table 3, Zovirax 5% ointment gave a 0% efficacy which was in good agreement with the theoretical C'* prediction. This lack of antiviral efficacy demonstrated by Zovirax 5% ointment is thus likely due to poor percutaneous ACV delivery from its polyethylene glycol vehicle (70, 71). The present results differ somewhat from those of Shannon et al. (72), who reported a significant reduction in the mean peak lesion score when the virus-infected hairless mice were topically treated with Zovirax 5% ointment. However, a detailed experimental procedure was not revealed by these authors and, therefore, a comparison between their results and those of the present study is impossible.

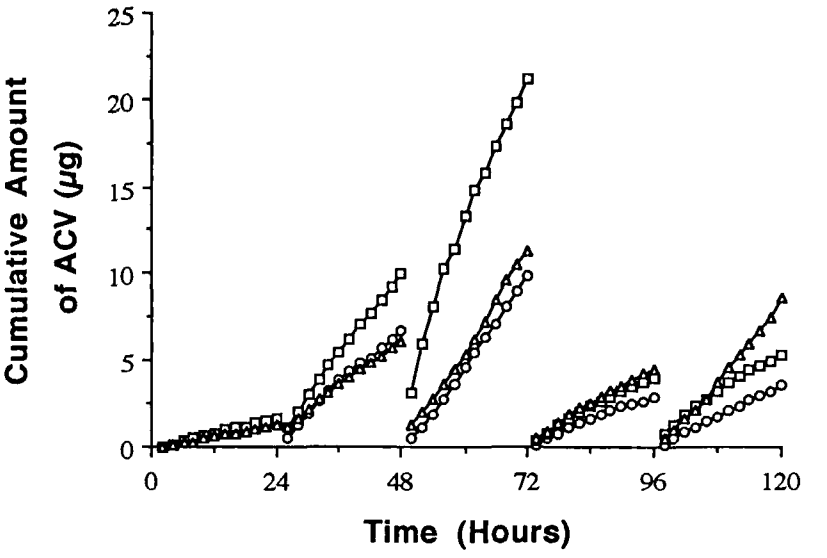

FIGURE 6

The accumulated amounts of ACV delivered topically across the hairless mouse skin into the receiver chamber of a Franz cell using a 2 μl finite dose 3% ACV suspension in a combined *in vivo-in vitro* experiment. The experiment was run in triplicate.

The skin samples treated with finite dose Zovirax 5% ointment gave the same general appearance as those untreated and it can be stated with confidence that the results presented in Table 3 were not complicated by the problem of skin irritation/damage.

In Vitro ACV Flux Determinations and *In Vivo* C_{ss}^{*} Assessments Using the Finite Dose 3% ACV Suspension

The procedures involved here were the same as those described above for Zovirax 5% ointment, except that a smaller (2 μl) finite dose was used for the self-prepared 3% ACV suspension. A finite dose of 3 μl or larger in the form of a suspension was more than what could be maintained on the skin surface of 1 cm^2. These higher doses were found to drain/drip from the application site; it was felt that if this were to happen *in vivo*, the antiviral efficacy results might be compromised by contact of the drug formulation with the inocultion site.

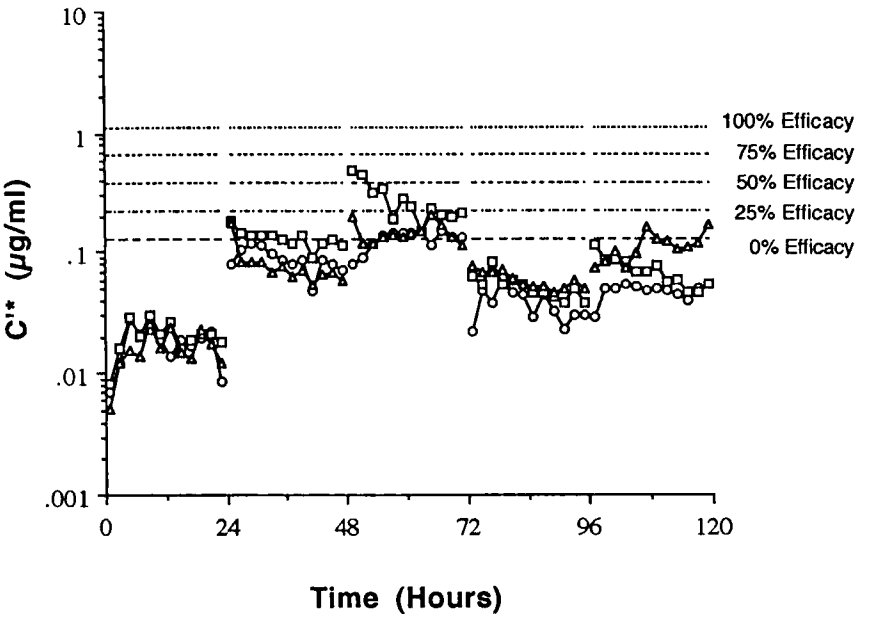

FIGURE 7

The temporal pattern of C'* assessed for 3% ACV suspension according to Eq.[7] using the instant ACV fluxes estimated from the slopes of the lines connecting the two consecutive time points in Fig. 6. The broken lines represent the C* values corresponding to the different antiviral efficacy calculated based on our previous results (69).

Figure 6 shows the accumulated amounts of ACV delivered topically across the hairless mouse skin into the receiver chamber of a Franz cell using finite dose (2 μl) 3% ACV suspension in a combined *in vivo-in vitro* experiment. Comparing these data to the Zovirax 5% ointment results, it is seen that the 3% ACV suspension demonstrated a somewhat larger variability over the five day experimental period, with the flux being the lowest on the first day, the highest on the third day, and about the same for the other three days. This same pattern was retained in Fig. 7 where the C'^* values calculated from the *in vitro* fluxes are presented. Excluding the first day results, the C'^* values for the remaining four days were found to be in the range from somewhere below that corresponding to a 0% topical efficacy to that for around a 25% topical efficacy.

In Vivo Antiviral Efficacy Studies Using the Finite Dose 3% ACV Suspension and Correlation Between the *In Vitro* Flux and the *In vivo* Efficacy Results

Table 4 summarizes the results of the first *in vivo* antiviral efficacy study obtained from the experimental group treated with the finite dose (2 μl) 3% ACV suspension and from the accompanying placebo and the control groups. The placebo group was treated with a (trans)dermal patch containing the aqueous Carbopol gel but without ACV. It is important to note that all the mice in the placebo group developed a "Th" lesion category and this validates our inoculation technique. The control group was treated with a (trans)dermal patch which used a 100% HEMA hydrogel membrane with 1 mm thickness to control the delivery of ACV. The ACV flux for this (trans)dermal patch was 422 μg/cm^2-day determined *in vitro* (31) and was 399 μg/cm^2-day estimated *in vivo* using the method of extraction (69). The control group showed a 100% topical efficacy and a 60% systemic efficacy and both results are consistent with our previous findings (69).

For the experimental group treated with the finite dose (2 μl) 3% ACV suspension in this first study, a 44% topical efficacy and à 44% systemic efficacy were obtained. These efficacy results were somewhat higher than those predicted. Based upon the flux data (Figure 6) and the C'* results (Figure 7), a topical efficacy of somewhere between 0% and 25% and a systemic efficacy of zero percent (66) would have been expected. The somewhat higher than predicted topical efficacy results may be still considered to be in satisfactory agreement with the C* prediction in view of statistics of small sample numbers; the rather higher degree of systemic efficacy (4 NR's) is however, more difficult to explain.

It was observed that the animals in the experimental group of the antiviral efficacy study (but not in the *in vivo-in vitro* flux study) exhibited a high frequency and a high degree of skin irritation and skin damage. This may be characterized by an erythema over the formulation application site in the early stage of the treatment and by dryness of the application site and, in the most severe cases, scabbing of the application site during the later stages. The cause(s) of this skin irritation was not investigated in the present study; however, the problem might be related to the presence in the formulation of 20% N-dodecyl-2-pyrrolidone which has been reported (73) to cause varying degrees of dermal irritation in rabbits depending on the concentration used. For the purpose of providing same measure of skin irritation/damage in the experiments, we have adopted an arbitrary scoring system which assigns a number from 0 to 4 to the various degrees of skin irritation/damage

TABLE 4

Results of the First In Vivo Antiviral Efficacy Study Using Finite Dose 3% ACV Suspension

Group	Lesion Category for Mouse No.										Antiviral Efficacy	
	1	2	3	4	5	6	7	8	9	10	Topical	Systemic
Placebo [a]	Th	Th	Th	Th	Th	Th	--[d]	Th	Th	Th	0 %	0 %
Control [b]	NR	St	NR	NR	NR	St	NR	St	St	NR	100 %	60 %
Experimental [c]	NR (1)[e]	NR (4)	NR (2)	Th (1)	Th (1)	Th (1)	Th (1)	NR (4)	Th (1)	--[d]	44 %	44 %

a. Treated with a (trans)dermal patch containing aqueous Carbopol gel but without ACV employing a two-day delayed three-day treatment protocol.
b. Treated with a (trans)dermal patch containing an ACV suspension employing a two-day delayed three-day treatment protocol. The (trans)dermal patch used a rate-controlling membrane made up of 100% HEMA with 1 mm thickness.
c. Treated with finite dose (2 μl) 3% ACV suspension every 12 hours employing a one-day delayed four-day treatment protocol.
d. The mouse died prior to the lesion scoring.
e. The number in the parenthesis under each lesion category in the experimental group was the corresponding skin irritation/damage score with 0 representing a nonirritative case and 4 being the most severe case.

with 0 being the nonirritative case and 4 representing the most severe case. For the animals in the *in vivo-in vitro* experiment, skin irritation was barely perceptible in the majority of the cases; most would score a 0 and some might score a 1 using this scoring system. For the animals in the *in vivo* efficacy study, the scores were generally higher and these are presented in Table 4 (within the parenthesis). It can be seen in Table 4 that only mouse no. 2 and mouse no. 8 showed severe (score 4) skin irritation; the other mice demonstrated slight (score 1) to mild (score 2) skin irritation. If the two mice with a severe skin irritation were excluded from the calculation of antiviral efficacy, the adjusted topical and systemic efficacy would both be 29% which would provide better agreement with the *in vitro* flux and C'*.

For the following reasons, it was decided to carry out a second antiviral efficacy study with the 3% ACV suspension. First, alerted to the problem of skin irritation/damage, we would be able to observe more closely this aspect in a second study. Also, it was thought prudent to run a repeat as the predictions versus actual results were less than ideal in the first study.

Table 5 presents the results of the second *in vivo* antiviral efficacy study with the self-prepared 3% ACV suspension. First, the placebo group showed zero efficacy: this again was assurance that the inoculation technique was reliable in this set of experiments. Secondly, the results for the control group (topical efficacy =90%; systemic efficacy = 20%) are in general agreement with those reported (69) in our earlier studies for the same rate-limiting membrane system (95% HEMA, 5% styrene; thickness = 0.5 mm).

For the experimental group treated with finite dose (2 μl) 3% ACV suspension, a 33% topical efficacy and a 22% systemic efficacy were observed. These values are not far from those predicted (predictions on the basis of *in vitro* flux and C*: topical efficacy = 0 to 25%; systemic efficacy = 0%). Again, skin irritation of varying degrees were observed in this experimental group. It is interesting to note that if mice with an irritation score of 4 are excluded, the adjusted topical efficacy would be 17% and the adjusted systemic efficacy would be 0% for the rest mice; these results would then be in very good agreement with predictions.

Assessment of the C'* Approach Based on the Action Mechanism of ACV

ACV exerts its antiviral effect on HSV-1 by interfering with viral DNA synthesis and thereby inhibiting viral replication (34,74). In *in vitro* cell culture

TABLE 5

Results of the Second *In Vivo* Antiviral Efficacy Study Using the Finite Dose 3% ACV Suspension

Group	Lesion Category for Mouse No.										Antiviral Efficacy	
	1	2	3	4	5	6	7	8	9	10	Topical	Systemic
Placebo [a]	Th	Th	Th	Th	Th	Th	Th	Th	Th	Th	0 %	0 %
Control [b]	J	St	Th	J	NR	St	St	NR	St	St	90 %	20 %
Experimental [c]	Th (1)[d]	Th (1)	Th (1)	J (2)	NR (4)	Th (3)	Th (4)	NR (4)	--[e]	Th (1)	33 %	22 %

a. Treated with a (trans)dermal patch containing aqueous Carbopol gel but without ACV employing a 2-day delayed 3-day treatment protocol.
b. Treated with a (trans)dermal patch containing an ACV suspension employing a two-day delayed three-day treatment protocol. The (trans)dermal patch used a rate-controlling membrane made up of 95% HEMA and 5% styrene with a thickness of 0.5 mm.
c. Treated with finite dose (2 μl) 3% ACV suspension every 12 hours employing a one-day delayed four-day treatment protocol.
d. The number in the parenthesis under each lesion category in the experimental group was the corresponding skin irritation/damage score with 0 representing a nonirritative case and 4 being the most severe case.
e. The mouse died prior to the lesion scoring.

studies, ACV is selectively phosphorylated by cells infected with HSV-1 to ACV monophosphate via virus-coded thymidine kinase; the monophosphate is subsequently converted to the diphosphate and the triphosphate via other cellular enzymes (75). *In vitro* antiviral studies (76, 77) have indicated that ACV triphosphate is the pharmacologically active form of the drug, the non-phosphorylated ACV, ACV monophosphate and ACV diphosphate are found to have minimal or no antiviral activity.

In this study, the concentration of the active drug species, ACV triphosphate, at the skin target site (the epidermal basal layer) was not determined. Instead, the ACV concentration (C'*) in the aqueous compartment bathing the epidermal basal cells was estimated from the *in vitro* flux (using eqn. [7]) and this was used for making predictions of the *in vivo* antiviral efficacy of ACV. This we believe is very reasonable and is based on the following arguments: (a) the uptake of ACV into human erythrocytes from the surrounding medium has been found (78) to take place very fast (equilibration time of the order of minutes) and we have no reason to believe that the *in vivo* transport of ACV into the epidermal basal cells of a hairless mouse would take place at greatly slower rates; (b) the extent of formation of ACV triphosphate in virus-infected cells is directly related to the concentration of ACV in the surrouding medium (75), and (c) it has been shown (79) that once the cells are deprived of their ACV source, the ACV triphosphate contents in the cells quickly decline with a half-life of 1-2 hours depending on the remaining concentration of ACV in the medium. The above arguments support the view that intracellular ACV triphosphate should likely be a single-valued function of C'* (for ACV) during a 3- or 4-day treatment period; consequently, the present approach of employing C'* as a predictor of *in vivo* efficacy appears to possess a sound foundation.

CONCLUSIONS

Despite the problem of skin irritation/damage which somewhat compromised the interpretation of our results, especially for the self-prepared formulation, a relatively good correlation was observed between the *in vitro* ACV flux (and the C'* predictions) and the *in vivo* antiviral efficacy in the present study. It is suggested that this good correlation validates the applicability of this hairless mouse

model in using the *in vitro* ACV flux and C'* to predict the *in vivo* antiviral efficacy.

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