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Research Paper

The Influence of Iontophoresis on Acyclovir Transport and Accumulation in Rabbit Ear Skin

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Purpose. The aim of this work was to explore the effect of iontophoresis on acyclovir (ACV) accumulation and permeation. In particular, the objectives were to check the efficacy of the transport mechanisms, electromigration and electroosmosis, on drug accumulation.
Methods. Permeation experiments were performed *in vitro*, using rabbit ear skin as barrier, from donor solutions at pH 3.0, 5.8, and 7.4. At the end of the experiments, drug accumulation in epidermis and dermis was measured. Anodal and cathodal iontophoresis were applied at pH 3.0, whereas only anodal iontophoresis was used at pH 5.8 (current densities 0.06–0.50 mA/cm^2) and 7.4.
Results. Cathodal iontophoresis was more efficient than anodal iontophoresis on ACV permeation across the skin at pH 3.0. At pH 5.8, ACV flux and accumulation increased with current density during anodal iontophoresis. At pH 7.4, anodal iontophoresis produced a remarkable increase of flux and a modest increase of accumulation. Overall, anodal flux increased as the pH of the donor solution was increased as a result of the increase of the skin net negative charge.
Conclusions. From the results obtained in the present work, it can be concluded that iontophoresis application increases ACV flux and, to a limited extent, accumulation in the skin.

KEY WORDS: acetaminophen; acyclovir; electroosmosis; iontophoresis; skin; transdermal.

INTRODUCTION

Acyclovir (ACV) is an antiviral agent used for the treatment of primary and recurrent labial and genital herpes, oral herpes, primary and recurrent varicella zoster, and herpes virus encephalitis (1). It is used orally, topically, or by infusion. The oral bioavailability of ACV is quite low (15 to 20%). The use of dermatological formulations of ACV, typically 5 or 10% creams or ointments, produced controversial efficacy in the treatment of recurrent infections in immunocompetent subjects (2). This poor efficacy was attributed to inadequate drug percutaneous penetration and to insufficient delivery of the drug to the target site of infection, the basal epidermis (3). Efforts aimed at increasing ACV transport/accumulation in the skin included the use of penetration enhancers, such as oleic acid in propylene glycol, (4) or vesicular systems, such as ethosomes (5). It was shown that ACV penetration and accumulation in human skin *in vitro* can be increased by using physical techniques, such as transdermal iontophoresis (6,7). The same technique was used *in vivo* in rabbits, and local skin concentrations were measured by microdialysis (8).

Iontophoresis is a promising technique for enhancing drug penetration across the skin by means of an externally applied electric current. This technique was reported to efficiently deliver opioids, nonsteroidal anti-inflammatory drugs, antivirals, local anesthetic, and so on (9). Although this technique is efficient primarily for ionized molecules, it was shown that it could increase the transport of nonionized molecules as well. In fact, one of the transport mechanisms, electroosmosis, drags all solutes present, regardless of their charge (10,11). The application of an electrical potential gradient across a charged porous membrane, such as the skin, produces a volume flow in the direction of the counterion transport (10), called electroosmotic flow. Electroosmotic flow in the anode-to-cathode direction results from the net negative charge supported by the skin at physiological pH (7.4) (12,13). The relative importance of electromigration and electroosmosis to the total flux of a molecule depends on its physicochemical properties, in particular on the molecular weight, because increasing the latter will decrease the electrical mobility, thus increasing the relative contribution of electroosmosis (8). However, no reports are available on the relative importance of electromigration and electroosmosis to drug accumulation in the skin.

The aim of this work was to explore the effect of iontophoresis on ACV accumulation and permeation. In particular, the objectives were to check the efficacy of the transport mechanisms involved, namely, electromigration and electroosmosis, on drug accumulation. Rabbit ear skin was used as barrier because it was shown to be a reasonable model for human skin *in vitro* (14–17).

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MATERIALS AND METHODS

Materials

Acyclovir (ACV; mol wt 225.2, pK_{a1} 2.4, pK_{a2} 9.2) was a gift from Lisapharma S.p.A. (Erba, Italy). Acetaminophen (AAP, mol wt 151.2) was obtained from ACEF (Fiorenzuola D'Arda, Italy) and HEPES from Sigma (St. Louis, MO, USA).

For high-performance liquid chromatography (HPLC) solvent preparation, distilled water and HPLC-grade acetonitrile were used. All other chemicals were of analytical grade.

Drug Analysis

Acyclovir

ACV analysis was performed by HPLC (Perkin Elmer, Norwalk, CT, USA), using a Vydac C18 250 × 4.6 mm column (Hesperia, CA, USA) and a mobile phase composed of distilled water at 1.2 mL/min (18). UV detection at 254 nm was used.

Acetaminophen

AAP analysis was performed by HPLC (Perkin Elmer), using a Waters C18 Novapak 150 × 3.9-mm column (Millipore Corporation, Milford, MA, USA) and a mobile phase composed of 10 mM sodium acetate (pH 4)–acetonitrile (60:40 v/v), at 1 mL/min. UV detection at 254 nm was used (17).

Permeation Experiments

Permeation experiments were conducted at 37°C in glass Franz-type diffusion cells (Disa, Milan, Italy; permeation area 0.6 cm^2). Rabbit skin was excised postsacrifice from the inner part of mixed-breed rabbit ears (6 months old) obtained from a local slaughterhouse. When not used immediately, the skin was kept refrigerated (2–5°C) and used within 3 days.

The donor compartment was filled with 1 mL of 25 mM HEPES-buffered saline at the following pH values:

1. pH 3.0, saturated with ACV (concentration 6.2 mM; 1.4 mg/mL)
2. pH 5.8, saturated with ACV (concentration 4.2 mM; 0.95 mg/mL)
3. pH 7.4, saturated with ACV (concentration 7.6 mM; 1.7 mg/mL)
4. pH 3.0, containing AAP (33 mM; 5 mg/mL).

The receptor compartment was filled with 4.3 mL of pH 7.4 phosphate-buffered saline, degassed under vacuum before use, and magnetically stirred to avoid boundary layer effects. At predetermined time intervals, 300 μL of receptor solution were sampled for analysis and replaced with the same volume of fresh solution. Phosphate-buffered saline at pH 7.4 was chosen as receptor medium to ensure a high solubility of ACV, and then to guarantee sink conditions to be respected.

In the iontophoretic experiments, the current was applied by means of a constant-current generator (Iono1, Cosmic, Pesaro, Italy), using silver/silver chloride electrodes made from silver wires (diameter 1 mm, purity 99.9%) and silver chloride (Sigma), in accordance with Green *et al.* (19). Direct current (0.06–0.5 mA/cm^2) was applied for 7 h. At the end of the experiment, the donor solution was removed, the cell was dismantled, and the skin was carefully washed to remove any residual donor solution. A disc of tissue was cut, fitting the area covered by the donor compartment (0.6 cm^2), and heated with a hair dryer for about 20 s; the epidermis was separated from the dermis with a scalpel (20). The two skin layers were placed in separate preweighed plastic test tubes, weighed again to determine the amount of tissue, and extracted with the appropriate solvent.

In the case of ACV, 500 μL of water were added to the skin sample and the extraction was conducted for 30 min at 60°C, vortexing occasionally. Then 500 μL of 1 M perchloric acid were added to precipitate proteins. After centrifugation for 10 min at 5,000 rpm and filtration with a 0.45-μm nylon filter, the extraction solution was analyzed by HPLC.

In the case of AAP, 300 μL of the mobile phase used for the HPLC analysis were added to the skin samples and the test tube was kept at 37°C for 1 h. After cooling, 100 μL of acetonitrile were added and the samples were centrifuged for 10 min at 11,000 rpm and filtered with a 0.45-μm nylon filter; the extraction solution was then analyzed by HPLC.

The extraction method was validated in blank experiments and by spiking the skin with a known amount of drug, either ACV or AAP. In all cases, no interfering peaks derived from the skin samples were detected. The recoveries are reported in Table I.

ACV skin concentration was calculated by normalizing the amount of ACV recovered in the epidermis and dermis by the total weight of the tissue (epidermis + dermis).

Statistical Analysis

All experiments were replicated six times; results were expressed as mean ± standard deviation. Statistical differences were determined by Kruskal–Wallis test.

RESULTS AND DISCUSSION

Iontophoresis at pH 3.0

ACV permeation across the skin was studied in conditions in which the drug is partly ionized, i.e., at pH 3.0, where the concentration of the positively charged form is 20% (5). Previous studies showed that anodal and, to a lower extent, cathodal iontophoresis (both applied for 7 h at 0.5 mA/cm^2) were able to produce a significant increase of ACV flux across nude mouse (5) and human skins (6). Anodal

Table I. Recoveries of Acyclovir and Acetaminophen from Epidermis and Dermis

	Epidermis recovery (%)	Dermis recovery (%)
ACV[a]	100.92 ± 1.21	99.14 ± 5.07
AAP[a]	99.96 ± 4.56	98.43 ± 4.41

Values are mean ± SD; $n = 6$.
[a] Amount spiked: 3 μg per sample.

iontophoresis was shown to increase ACV concentration in human skin as well. In this work, we decided to use a lower current density, namely, 0.125 mA/cm^2, to reduce the chance of producing significant alterations of skin permeability.

The penetration profiles of ACV across rabbit ear skin, obtained after the application of anodal and cathodal iontophoresis at 0.125 mA/cm^2 for 7 h, are reported in Fig. 1a, together with the passive profile. The flux values, calculated as the slope of the regression line in the interval 3–7 h, are reported in Fig. 2a. Passive diffusion generated very small amounts of ACV that penetrated and, surprisingly, cathodal iontophoresis was more efficient than anodal iontophoresis ($p < 0.01$ in Fig. 2a) in promoting ACV penetration across the skin, although ACV was positively charged at pH 3.0. At this pH value, ACV can be transported by electrorepulsion in the anode-to-cathode direction, and—if the charge of the skin is reversed—by electroosmosis in the cathode-to-anode direction. The isoelectric point of mammalian skin was reported as being included in the interval 3–5 (21–23); thus, pH 3.0 corresponds to a reversal of skin charge. ACV data are consistent with previously published data on the permselective properties of rabbit ear skin (14), where it was shown that, using sodium transport number, the isoelectric point of rabbit ear skin resulted in the interval 2–3. In the specific case

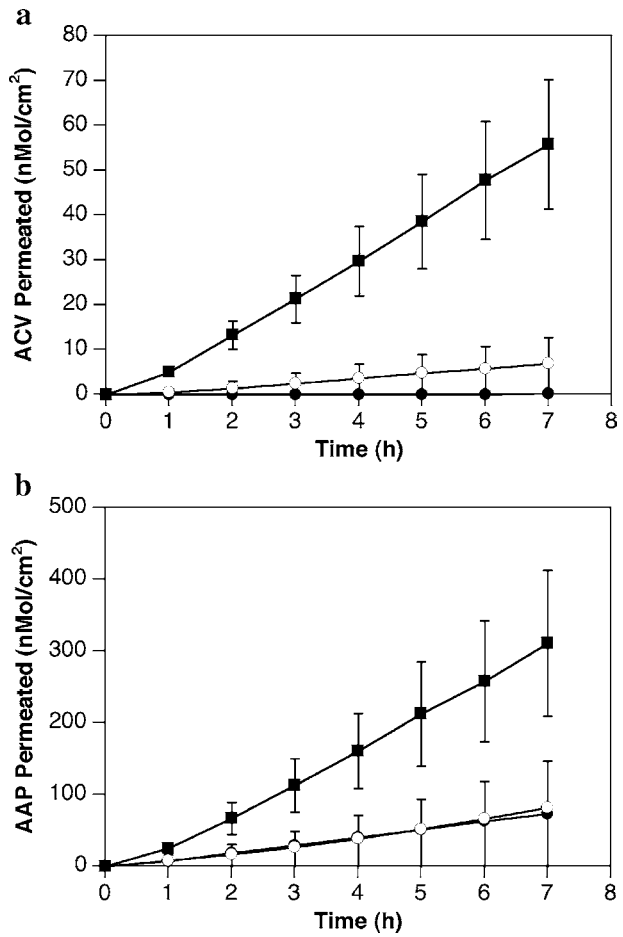

Fig. 1. (a) Acyclovir and (b) acetaminophen permeation profiles across rabbit ear skin during passive diffusion (●), anodal iontophoresis (○), and cathodal (■) iontophoresis at pH 3.0 and current density 0.125 mA/cm^2. Mean ± SD.

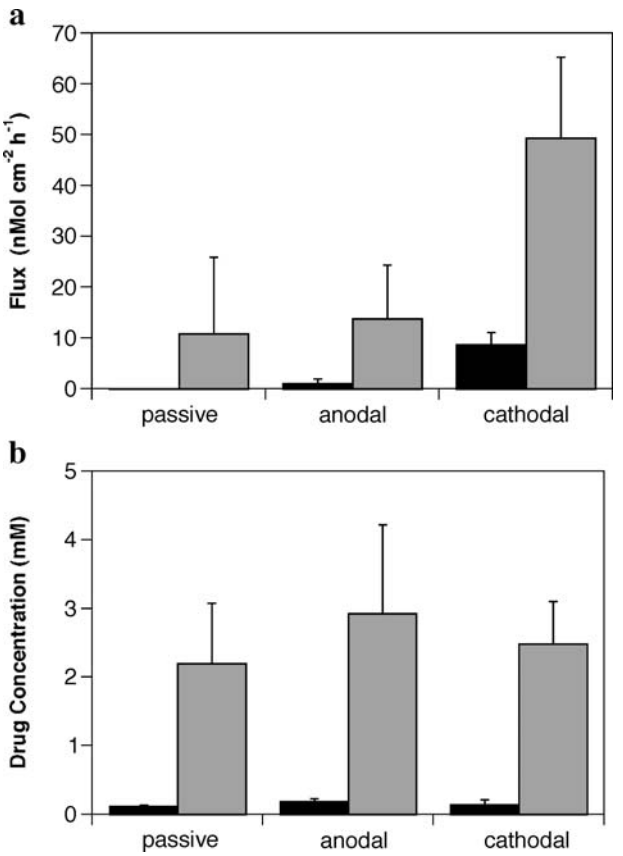

Fig. 2. (a) Average flux and (b) skin concentration of acyclovir (dark bars) and acetaminophen (light bars) after 7 h of passive diffusion, anodal iontophoresis, and cathodal iontophoresis (0.125 mA/cm^2) at pH 3.0. Mean ± SD.

of ACV at pH 3.0, it seems that during anodal iontophoresis, electrorepulsion is counterbalanced by electroosmotic flow, which is in the opposite direction. However, the data of Volpato *et al.* (5) on nude mouse skin report a higher anodal transport compared to cathodal. The differences between the cited experiments and those reported in the present work, besides the skin type, are current density and buffer solution composition. In particular, Volpato *et al.* (5) used higher current density (0.5 mA/cm^2) and higher ionic strength of the donor solution (0.24 vs. 0.14 M). It was shown that electrorepulsion and electroosmosis decrease with increasing ionic strength, although the sensitivity of the two transport mechanisms to increasing ionic strengths might not be the same. On the other hand, electrorepulsion and electroosmosis increase with current density, although it was suggested (5) that electrotransport of ACV is more dependent on current density than electroosmotic transport.

To quantify the electroosmotic contribution to ACV transport, the same experiment done on ACV was repeated on a hydrophilic nonionized molecule such as AAP, which, being totally nonionized at pH 3.0, can only be transported by electroosmosis. In addition, AAP is easily determined by HPLC, thus avoiding the use of radioactive tracers, such as the case of mannitol or glucose. The results obtained, reported in Fig. 1b, and the relative fluxes reported in Fig. 2a show that the trend of anodal and cathodal iontophoresis in

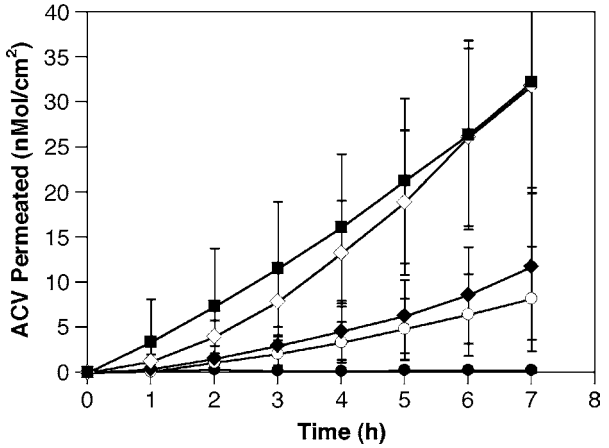

Fig. 3. Acyclovir permeation profiles across rabbit ear skin during passive diffusion (●), and anodal iontophoresis at 0.06 (○), 0.125 (◆), 0.25 (◇) and 0.50 (■) mA/cm^2 at pH 5.8. Mean ± SD.

AAP transport across the skin was the same as for ACV, although in the former there was no difference between passive and anodal flux. Because AAP is not ionized, its flux represents precisely the magnitude of electroosmotic flow. The prevalence of cathodal over anodal flux confirms the net positive charge of rabbit ear skin at pH 3.0. From AAP fluxes, the corresponding water volume flows were calculated according to Marro *et al.* (22), and the results were 0.33 ± 0.45, 0.42 ± 0.32, and 1.49 ± 0.48 μL cm^{-2} h^{-1} for passive flux, anodal iontophoresis, and cathodal iontophoresis, respectively. It was then possible to calculate the relative contribution of electroosmosis and electrorepulsion to ACV transport across rabbit ear skin, according to Marro *et al.* (22). The calculations showed that electroosmosis accounts for 52% of anodal flux and 96% of cathodal ACV flux. Due to the low percentage of ACV in the ionized form and/or to the low current density used, electroosmosis predominates over electrorepulsion in both anodal and cathodal iontophoresis.

The corresponding amounts of ACV and AAP accumulated in the skin are listed in Table II. Epidermal accumulation of ACV was fairly constant for passive flux and anodal and cathodal iontophoresis, whereas dermal accumulation increased with the application of current, in particular when cathodal iontophoresis was used (p = <0.01). In the case of AAP, the trend was the same, although the differences in dermal accumulation were not statistically significant. In general, AAP accumulates to a greater extent than ACV, owing to the higher concentration in the donor compartment and probably to different partitioning. Since the weight of the single skin specimen can be slightly different, ACV and AAP accumulation data were transformed in concentration, by dividing the cumulative amount of drug recovered in epidermis + dermis by the total weight of the skin sample. This value of concentration represents the average concentration of the drug in the skin tissue. The results obtained are reported in Fig. 2, together with the corresponding average fluxes. For both drugs, skin accumulations were almost constant in the three conditions examined, whereas flux increased in the order passive flux, anodal iontophoresis, and cathodal iontophoresis. In particular, for ACV, anodal and cathodal fluxes were significantly different between them and from passive flux, whereas for AAP, passive and anodal fluxes were not significantly different.

Overall, these results indicate that the flux across the skin was more sensitive to current application than the corresponding concentration of the active in the skin tissue. If one considers in particular cathodal iontophoresis, where the main mechanism is electroosmosis, it seems like electroosmotic solvent flow can produce a significantly higher flux *across* the skin but cannot change significantly the concentration of the active *inside* the skin. It is possible that the extent of accumulation depends on the partitioning characteristics of the molecule. ACV and AAP show negative values of log P (octanol/water partition coefficient), −1.57 for ACV (24) and −0.90 for AAP (25), indicating that they are both hydrophilic molecules and so do not have the tendency to migrate outside the "pores" of the skin, through which the electroosmotic flow was shown to take place (26).

Iontophoresis at pH 5.8

Permeation experiments were also performed at pH 5.8, which corresponds to the isoelectric point of ACV, where the molecule does not bear a net electric charge. Only anodal iontophoresis was used. Figure 3 reports the permeation profiles obtained by applying increasing current densities, from 0.06 to 0.5 mA/cm^2, together with the no-current

Table II. ACV and AAP Accumulation Data in Epidermis and Dermis

pH	Condition	Current density (mA/cm^2)	Amount in epidermis (nmol)		Amount in dermis (nmol)	
			ACV	AAP	ACV	AAP
3.0	Passive	0	2.71 ± 0.98	23.02 ± 9.46	0.90 ± 0.55	18.93 ± 4.52
3.0	Anodal	0.125	2.46 ± 1.07	38.19 ± 19.13	1.61 ± 1.04	26.72 ± 10.79
3.0	Cathodal	0.125	2.92 ± 0.98	35.33 ± 10.73	3.18 ± 0.80*	32.30 ± 14.20
5.8	Passive	0	1.48 ± 0.91		1.79 ± 1.06	
5.8	Anodal	0.06	1.65 ± 0.75		1.12 ± 0.43	
5.8	Anodal	0.125	2.26 ± 0.56		1.42 ± 0.49	
5.8	Anodal	0.25	3.45 ± 1.97		2.18 ± 0.95	
5.8	Anodal	0.5	4.35 ± 1.95*		3.36 ± 2.00	
7.4	Passive	0	1.80 ± 0.22		2.98 ± 1.70	
7.4	Anodal	0.125	3.20 ± 1.13		3.42 ± 1.24	

Values are mean ± SD.
* $p < 0.05$, significantly different from the corresponding passive value.

control. Passive diffusion of ACV across rabbit ear skin at pH 5.8 was very close to zero, whereas with increasing current density measurable amounts of ACV permeated the skin. The increase of skin penetration of ACV with current density was roughly proportional, although no differences were found between 0.06 and 0.125 mA/cm^2 and between 0.25 and 0.5 mA/cm^2. At pH 5.8 ACV is not ionized, thus the transport mechanisms involved should be passive diffusion and electroosmosis. Passive diffusion gives a very low contribution to drug transport, so the prevailing enhancing mechanisms is electroosmosis.

The corresponding epidermis and dermis accumulation data are reported in Table II, where it can be observed that ACV accumulation increases with current density in both epidermis and dermis. The absolute amounts of ACV accumulated were typically slightly higher for epidermis compared to dermis, although the difference was not statistically significant. Compared with the corresponding results obtained at pH 3.0, the differences are again not significant. ACV accumulation data were then transformed in concentration, by dividing the total amount of ACV recovered by the weight of tissue (epidermis + dermis). When ACV skin concentration was plotted as a function of current density applied (Fig. 4), it increases stepwise, and, in particular, the effect of 0.25 mA/cm^2 is the same as that of 0.5 mA/cm^2. The same trend is shown by the corresponding flux values, indicating that current application had the same effect on flux and accumulation.

Iontophoresis at pH 7.4

At pH 7.4, ACV is present basically in the un-ionized form (6), but the skin bears a net negative charge (14). ACV passive skin accumulation at pH 7.4, reported in Table II, was similar compared with pH 3.0 and 5.8. The application of anodal iontophoresis (0.125 mA/cm^2) produced a slight increase of ACV accumulation in epidermis and dermis. Concerning ACV transport across the skin, as expected from the negative charge of the skin the flux increased significantly compared with the other pH values (data not shown).

Normalized fluxes of ACV, i.e., flux values divided by the corresponding drug donor concentration, were calculated and are reported in Table III. Whereas passive normalized fluxes were close to or equal to zero for all pH values tested, anodal iontophoretic flux increased with increasing pH value of donor solution as the result of the progressive increase of skin negative charge.

Relationship Between Flux and Accumulation

Finally, ACV flux values were plotted vs. the respective ACV skin concentrations and the result is shown in Fig. 5. The figure reports all individual values obtained in the various experimental conditions of pH and current application. It can be observed that despite the scattering of the data, ACV flux shows the tendency to increase with skin concentration, although a clear trend cannot be seen. There is apparently a minimum ACV skin concentration below which no measurable flux is observed: above this value, flux tends to increase with skin concentration. Statistical evaluation revealed that there was correlation between flux and skin concentration and the regression line resulted: ACV flux = −1.78 (95% confidence interval −3.09 to −0.47) + 26.84 (95% confidence interval 20.60–33.09) × ACV concentration (R=0.767).

CONCLUSIONS

From the results obtained in the present work, it can be concluded that iontophoresis application increases ACV skin flux and, to a limited extent, ACV skin accumulation. In particular, skin concentration remained almost constant during passive diffusion and anodal and cathodal iontophoresis (0.125 mA/cm^2) at pH 3.0 for both ACV and AAP. ACV and AAP fluxes increased primarily during cathodal iontophoresis; for ACV, which is partially in the ionized (positive) state, anodal flux was also slightly increased. Anodal iontophoresis at pH 5.8 produced an increase of both flux and skin concentration of ACV, with increasing current densities. The use of a pH 7.4 donor solution remarkably increased ACV anodal flux due to the electroosmotic contribution to drug transport and, to a limited extent, also ACV skin accumulation.

Overall, ACV skin flux was clearly governed by the direction and extent of electroosmotic flow, whereas skin accumulation seemed to be less sensitive to the application of

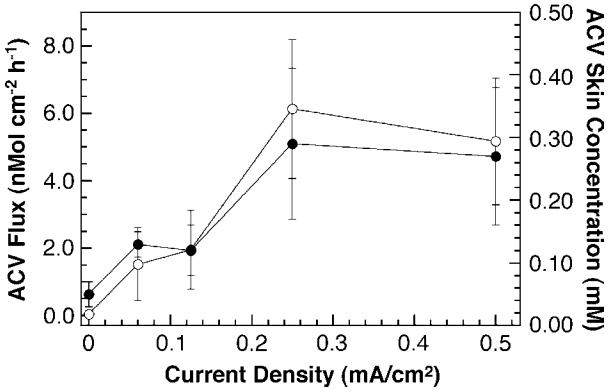

Fig. 4. Relationship between flux (○) and skin concentration (●) with current density during anodal iontophoresis at pH 5.8. Mean ± SD.

Table III. Normalized Fluxes of ACV During Iontophoresis

Current density (mA/cm^2)	Normalized flux (cm/h) × 10^4		
	pH 3.0	pH 5.8	pH 7.4
0	0.04 ± 0.08	0.09 ± 0.12	0
Anodal			
0.06	–	3.62 ± 2.59	–
0.125	1.77 ± 1.44^{a,b}	4.65 ± 2.78^{a}	11.19 ± 5.42^{a}
0.25	–	14.47 ± 4.96	–
0.50	–	12.30 ± 4.47	–
Cathodal			
0.125	14.01 ± 3.90^{b}	–	–

Values are mean ± SD. Values with superscript letters are significantly different from each other ($p < 0.05$).

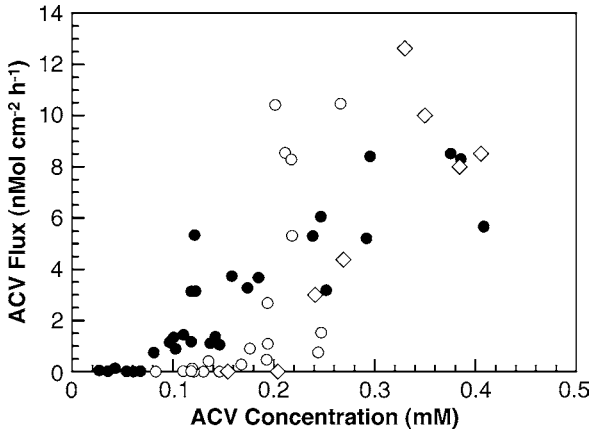

Fig. 5. Relationship between individual values of flux and skin concentration at pH 3.0 (○), 5.8 (●) and 7.4 (◇).

an electric field. When ACV fluxes were plotted against the respective skin concentrations, ACV flux showed the tendency to increase with skin concentration, although a clear trend cannot be seen.

The results of this work cannot be transported directly to the *in vivo* situation because the experiments were performed *in vitro* on nonviable animal skin, but offer some insights on the relative importance of electroosmosis and electrorepulsion on ACV transport into and across the skin at different pH values.

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