In Vivo and *In Vitro* Percutaneous Absorption of Cancer Preventive Flavonoid Apigenin in Different Vehicles in Mouse Skin[1]

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Purpose. *In vivo* and *in vitro* percutaneous absorption of apigenin was investigated in three vehicles previously used in cancer prevention studies to determine the drug delivery properties for optimal chemopreventive activity.

Methods. *In vivo* percutaneous absorption of apigenin on SENCAR mice was studied with DMSO and acetone/DMSO (A/D, 4:1) vehicle. *In vitro* percutaneous absorption studies used whole mouse skin, without subcutaneous fat, mounted on Franz diffusion cells with 37°C Dulbecco's phosphate-buffered saline as the receptor fluid. The skin was treated with [G-^{3}H]-apigenin in DMSO, A/D (4:1), or propylene glycol/DMSO (PG/D, 4:1).

Results. Apigenin uptake by epidermal cells and distribution in epidermis following *in vivo* topical treatment in two vehicles was in the order of A/D > DMSO, while apigenin distribution in dermis and subcutaneous fat was not different between DMSO and A/D. Total apigenin absorption in mouse skin *in vitro* was in the order of A/D > DMSO > PG/D. However, apigenin sub-tissue distribution within epidermis determined by tape-stripping and by determination of apigenin in dermal and epidermal tissue indicated that DMSO delivered more apigenin into viable epidermis than A/D while A/D deposited more apigenin in the stratum corneum. Apigenin absorption in mouse skin with DMSO or A/D showed saturation kinetics while apigenin in PG/D showed very low absorption initially and non-saturated absorption in a period of 6 hr. HPLC-scintillation profiles of *in vitro* samples showed no evidence of apigenin metabolism in mouse skin.

Conclusions. Delivering apigenin into viable epidermis appears to be a necessary property for an apigenin formulation to be effective in skin cancer prevention.

KEY WORDS: apigenin; flavonoid; percutaneous absorption; solvent effect; tape-stripping.

INTRODUCTION

Apigenin (4',5,7-trihydroxyflavone), a plant flavonoid, inhibited skin tumorigenesis when applied topically in dimethylsulfoxide (DMSO) but showed no inhibition in acetone/DMSO (A/D, 9:1) (1, 2). Apigenin in three vehicles inhibited ornithine decarboxylase (ODC) induction by 12-O-tetradecanoylphorbol-13-acetate (TPA) in the order of DMSO > A/D (4:1) > propylene glycol/DMSO (PG/D, 4:1) (1). These vehicle effects might be the results of differential delivery of apigenin to the target site by these vehicles. In this study, we tested this hypothesis by correlating drug delivery characteristics of these vehicles with the vehicle effects observed in chemoprevention. Thus, this study is to characterize the *in vivo* and *in vitro* percutaneous absorption of apigenin in previously used vehicles. For *in vivo* absorption, we compared apigenin uptake in epidermal cells and distribution in mouse skin using DMSO and A/D (4:1) vehicles. For *in vitro* absorption, we compared apigenin absorption in skin sections and receptor fluid among DMSO, A/D (4:1), and PG/D (4:1) vehicles.

MATERIALS AND METHODS

Chemicals

Apigenin was obtained and purified as described previously (1). [G-^{3}H]-apigenin was prepared and purified as described elsewhere (3). Acetone and DMSO were purchased from Mallinckrodt, Inc., Paris, KY. Propylene glycol, trypsin, and trypsin inhibitor was purchased from Sigma Chemical Company, St. Louis, MO.

Animals

Female SENCAR mice (6 weeks old) were obtained and maintained as described previously (1). The research adhered to the "Principles of Laboratory Animal Care" (NIH publication #85-23).

In Vivo Apigenin Uptake in Mouse Epidermal Cells

The mice (8–10 weeks old) were shaved on the back 24 hr before treatment and caged individually. A plastic ring (1.5 cm in diameter, 1 cm wide) was put on animal's necks to restrict them from licking the drug. DMSO or A/D (4:1)(100 μl) with or without a given dose of apigenin was applied to the dorsal skin (4 cm^2 area). At the specified time, the mice were killed, and the drug-treated skin area was washed with 5 × 200 μl 0.1% Tween 20 solution and rinsed with 3 × 200 μl water. The skin washes were combined and sampled to quantitate apigenin UV spectrometrically at 337 nm.

The drug-treated area was cut from the mouse, and the subcutaneous fat was removed with a scalpel. The skin was incubated for one hour at 37°C with the dermal side on 0.5% trypsin (pH = 7.4; with 1 mM EDTA) to separate epidermis from dermis. Epidermis was scraped from the dermis and cells were isolated by cutting and briefly stirring the epidermis in Dulbecco's phosphate-buffered saline (D-PBS, pH 7, with 0.01% trypsin inhibitor). After filtration, cell counting, and centrifugation, the cell pellet was homogenized in absolute ethanol to extract apigenin using a Polytron homogenizer (Brinkmann Instruments, Westburry, NY). Apigenin in the extract was then quantitated by HPLC as described elsewhere (3). The epidermal debris and dermis were individually extracted with ethanol, and apigenin was quantitated UV spectrometrically at 337 nm.

In Vivo Apigenin Distribution in Mouse Skin

Four hours after apigenin treatment the skin was washed as described above, and separated into epidermis, dermis and

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fat layers by scraping (epidermis separation was facilitated with heatshock at 57°C for 30 seconds). These skin sections were homogenized in absolute ethanol, and apigenin in the extracts was quantitated UV spectrometrically at 337 nm using ethanol extract from vehicle control as the blank.

In Vitro Percutaneous Absorption Through Mouse Skin

The whole mouse dorsal skin without subcutaneous fat was cut into pieces and mounted on separate Franz diffusion cells (1.0 cm diameter) filled with 37°C D-PBS that maintains skin viability for over 24 hr (4). The skin was treated with [G-^{3}H]-apigenin in DMSO, A/D (4:1), or PG/D (4:1) (10 μl, 25 mM, 13–17 μCi/μmole). The receptor fluid was either sampled at predetermined time points during the absorption (continual method) or sampled after terminating the absorption at specified time points (separate method). Radioactivity was assayed by scintillation counting using EcoLume scintillation fluid (ICN, Costa Mesa, CA).

At the specified times, the skin was removed from the Franz cell and washed with 0.1% Tween 20 as described earlier (skin wash was sampled to quantitate radioactivity). The skin surrounding the drug-treated area was trimmed (surrounding skin), and the epidermis and dermis of the drug-treated area were separated by scraping. These skin sections were individually solubilized in NCS tissue solubilizer (Amersham Corp., Arlington Heights, IL) and assayed for radioactivity by scintillation counting. The percentage of radioactivity associated with apigenin in epidermis, dermis, skin wash, and receptor fluid was determined by HPLC-scintillation analysis as described below.

In Vitro Tape Stripping

Following *in vitro* absorption and skin wash, the skin was fixed on a self-designed tissue holder with a circular opening on the upper piece (1.2 cm diameter). The drug-treated skin area was then stripped with polypropylene tape (Scotch Book Tape No. 845, 3M) that could effectively remove the stratum corneum (SC) of SENCAR mice as tested by the permeation of [G-^{3}H]-apigenin through tape-stripped skin and by histologic examination. The tape was weighed before and after stripping (3–5 replicates were pooled to provide adequate tissue for weighing). The tape strips were held in scintillation fluid for over 24 hr to release radioactivity before scintillation counting. The remaining skin was cut into drug-treated skin (skin w/o SC) and surrounding skin, and individually solubilized in NCS tissue solubilizer for radioactivity counting.

HPLC-Scintillation Analysis

Epidermis and dermis were extracted with absolute ethanol. Apigenin in receptor fluid was extracted with ethyl ether, and re-dissolved in absolute ethanol after evaporating ethyl ether. Samples were filtered through 0.2 μm membrane and injected into HPLC system equipped with Alltima C18 column (2.1 × 250 mm, Alltech). The column was eluted with 0.1% TFA in water/acetonitrile (52:48) at 0.3 ml/min. The eluent was collected as 0.5 min fractions and assayed for radioactivity by scintillation counting.

Data are reported as mean ± SE.

RESULTS

In Vivo Apigenin Uptake in Mouse Epidermal Cells

The time course and dose response of *in vivo* apigenin uptake in epidermal cells are summarized in Table I. The zero hour data were collected right after drug application and used as controls for drug recovery and background absorption. Apigenin concentration in epidermal cells was generally higher with A/D (4:1) than with DMSO ($p < 0.01$ by Anova) although the difference was not significant at the high dose (5.0 μmol). Drug recovery was in 63–99% range and not different between the two groups ($p = 0.7$ by Anova). The lower recoveries after 1 hr were primarily due to drug lost through transfer from the mice to the cage wall, but there was probably some absorption into the body. Both groups showed increased apigenin uptake with higher doses ($p < 0.05$ by Anova).

In Vivo Apigenin Distribution in Mouse Skin

The *in vivo* apigenin distribution in mouse epidermis 4 hr after topical application in two vehicles was in the order of A/D > DMSO (378 ± 12 vs. 156 ± 25 pg/μg tissue, $p < 0.01$ by *t*-Test) while the distribution in dermis and fat layer was not different between the two groups ($p > 0.05$ by *t*-Test). Drug recovery was not different between A/D and DMSO (90 ± 1 vs. 87 ± 4%, $p > 0.2$).

In Vitro Apigenin Distribution in Mouse Stratum Corneum

Radioactivity distribution in SC was determined by tape-stripping after treatment with [G-^{3}H]-apigenin in DMSO, A/D (4:1), and PG/D (4:1) for 4 hr. The SC weight removed by tape-strippings was not different with the three vehicles ($p > 0.1$

Table I. Apigenin Uptake in Epidermal Cells Following *In Vivo* Percutaneous Absorption in SENCAR Mice[a]

	Apigenin 10^{-17} mol/cell (%recovery)[b]		*p* (*t*-Test)
	DMSO	A/D	
Time Course at 2.5 μmol dose			
0 hr	2.2 ± 0.3 (98 ± 4)	2.5 ± 0.1 (99 ± 5)	0.3
0.5 hr	6.8 ± 1.1 (88 ± 5)	20.4 ± 2.4 (98 ± 2)	0.02
1 hr	12.3 ± 4.4 (86 ± 11)	22.8 ± 4.3 (63 ± 3)	0.04
2 hr	13.9 ± 2.5 (73 ± 8)	24.8 ± 5.0 (75 ± 3)	0.04
4 hr	14.6 ± 5.0 (75 ± 6)	33.0 ± 3.4 (79 ± 7)	0.001
6 hr	15.3 ± 3.0 (75 ± 3)	31.0 ± 5.0 (67 ± 4)	0.03
Dose Response at 4 hr			
1.25	7.1 ± 1.3 (76 ± 14)	17.7 ± 3.2 (81 ± 2)	0.02
2.5	14.6 ± 5.0 (75 ± 6)	33.0 ± 3.4 (79 ± 7)	0.04
5.0	44.2 ± 18 (82 ± 6)	49.5 ± 15 (70 ± 12)	0.30

[a] The mice were treated with apigenin at 1.25, 2.5, and 5.0 μmol dose in 0.1 ml solvent (12.5, 25, or 50 mM) on 4 cm^2 of dorsal skin. Epidermal cells were isolated by trypsin digestion. A/D = acetone/DMSO (4:1).

[b] Apigenin detected by HPLC-UV and expressed as Mean ± SE (n = 3 mice for each value). Apigenin recovery was calculated from the sum of apigenin recovered from epidermal cells, epidermal debris, dermis, and skin wash.

by Anova) (Figure 1A). Radioactivity retention was generally in the order of A/D > DMSO > PG/D in the outer layers of SC while it was not different in the inner layers (Figure 1B). Compared with DMSO, the radioactivity was significantly higher with A/D in the first three individual strippings ($p < 0.05$ by t-Test). Compared with PG/D, the radioactivity in SC was greater with DMSO only in the first tape-stripping ($p < 0.05$ by t-Test).

In Vitro Percutaneous Absorption of Apigenin in Mouse Skin

Figure 2 shows the radioactivity in SC (removed by 10 tape-strippings), skin w/o SC, receptor fluid, and total absorp-

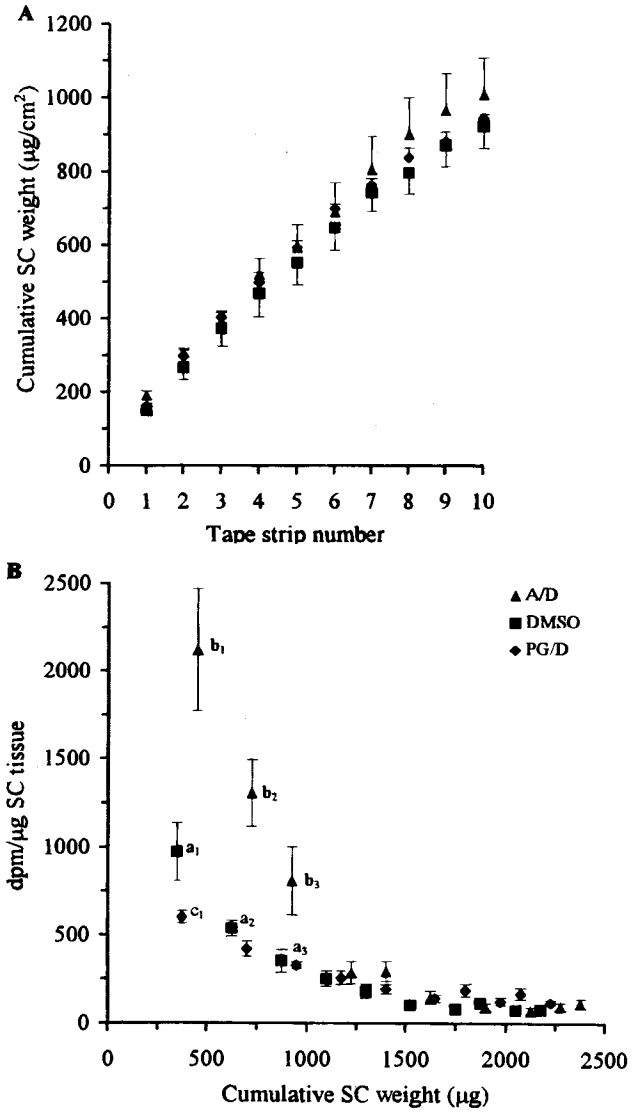

Fig. 1. Stratum corneum (SC) weight removed by tape-stripping (A), and distribution of radioactivity in SC (B) after *in vitro* absorption of apigenin in three vehicles spiked with [G-^{3}H]-apigenin (10 μl, 25 mM, 16.7 μCi/μmole) through mouse skin for 4 hr. Three replicate tape-strippings were pooled to provide adequate tissue for weighing. The SC tissue removed was not different by weight among the three vehicle groups ($p > 0.1$ by Anova). In figure B, $b_1 > a_1$, $b_2 > a_2$, $b_3 > a_3$, $a_1 > C_1$ ($p < 0.05$ by t-Test). The data are expressed as Mean ± SE ($n = 4$).

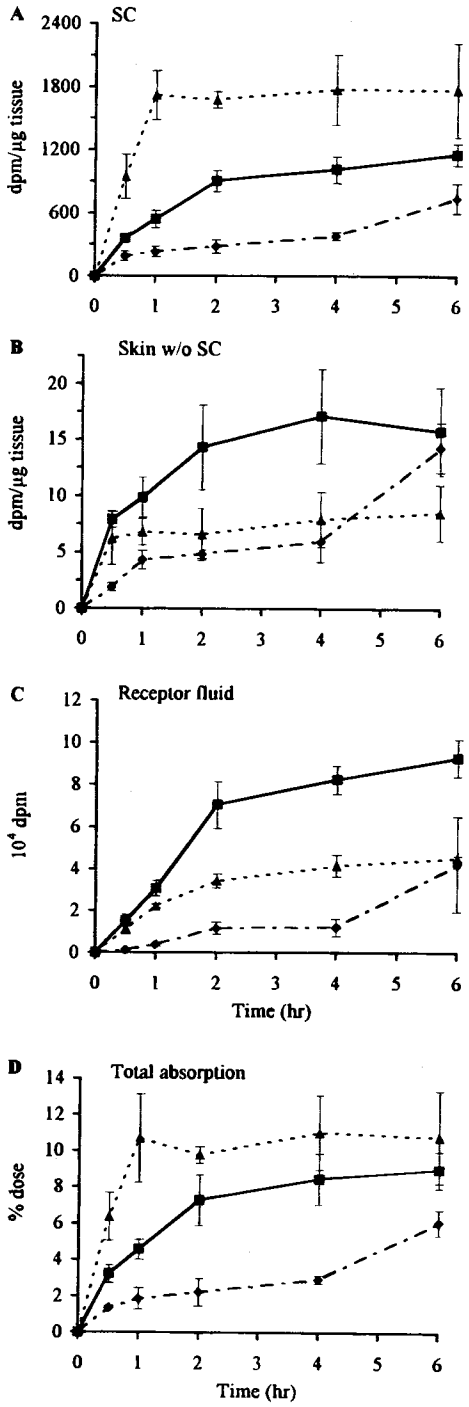

Fig. 2. *In vitro* percutaneous absorption of apigenin in three vehicles spiked with [G-^{3}H]-apigenin (10 μl, 25 mM, 16.7 μCi/μmole) through mouse skin. All the data were measured separately from different diffusion cells by terminating absorption at a specified time point. Data are expressed as Mean ± SE ($n = 4$). (■) DMSO; (▲) A/D; (◆) PG/D. A. Radioactivity in stratum corneum (SC). Radioactivity order: A/D > DMSO > PG/D at 0.5–6 hr ($p < 0.05$ by t-Test). B. Radioactivity in skin w/o SC. Radioactivity order: DMSO > A/D at 1–6 hr ($p < 0.05$); A/D > PG/D at 0.5–1 hr ($p < 0.05$). C. Radioactivity in receptor fluid. Radioactivity order: DMSO > A/D at 1–6 hr ($p < 0.05$); A/D > PG/D at 0.5–4 hr ($p < 0.005$). D. Total radioactivity absorbed (the sum of radioactivity in SC, skin w/o SC and receptor fluid), expressed as the percentage of dose applied. Radioactivity order: A/D > DMSO at 0.5–2 hr ($p < 0.05$); DMSO > PG/D at 0.5–6 hr ($p < 0.02$).

Table II. Distribution of Apigenin Recovered Following *In Vitro* Percutaneous Absorption for 4 hr in Mouse Skin[a]

Section	DMSO			A/D			PG/D		
	ng	%dose	%abs	ng	%dose	%abs	ng	%dose	%abs
SC	372 ± 46	5.5	65	650 ± 123	9.6	87	141 ± 13	2.1	71
Skin w/o SC	140 ± 34	2.1	25	65 ± 20	1.0	9	49 ± 15	0.7	25
Receptor fluid	55 ± 4	0.8	10	28 ± 3	0.5	4	8 ± 3	0.1	4
Total absorbed	566 ± 84	8.5	100	743 ± 147	11.0	100	198 ± 31	2.9	100
Surround skin	59 ± 19	0.9		42 ± 4	0.6		62 ± 18	0.9	
Skin wash	6283 ± 174	92.9		5967 ± 430	88.3		6371 ± 301	94.3	
Total recovered	6908 ± 277	102.3		6752 ± 581	99.9		6631 ± 350	98.1	

[a] The mouse skin w/o subcutaneous fat mounted on Franz cells was treated apigenin solutions spiked with [G-^{3}H]-apigenin (10 μl, 25 mM, 16.7 μCi/μmole). Apigenin quantities (ng) are calculated from radioactivity data and expressed as Mean ± SE (n = 4). "Total absorbed" data are the sum of SC, Skin w/o SC and Receptor fluid. "Total recovered" data are the sum of total absorbed, surround skin, and skin wash. %dose = percentage over the dose applied; %abs = percentage over total absorbed.

tion 0.5–6 hr after *in vitro* treatment with [G-^{3}H]-apigenin. Generally, radioactivity in SC is in the order of A/D > DMSO > PG/D while the order in skin w/o SC and receptor fluid is DMSO > A/D > PG/D ($p < 0.0001$ by Anova) although the difference was not significant at some time points. Total absorption was in the order of A/D > DMSO > PG/D ($p < 0.0001$) while the difference between DMSO and A/D was not significant at 4–6 hr ($p > 0.2$ by *t*-Test). During the absorption process, the receptor compartment remained at sink conditions for apigenin absorption (apigenin concentration ≤ 0.14 μg/mL, 10% of apigenin solubility).

The kinetic characteristics of the *in vitro* percutaneous absorption were also different among the three groups. DMSO and A/D groups showed saturation kinetics with radioactivity in SC (Figure 2A), skin w/o SC (Figure 2B), and total absorption (Figure 2D). Compared with DMSO group, A/D group reached the maximum sooner. In contrast, PG/D group showed very low absorption initially and nonsaturated absorption kinetics in the 6 hr period.

Table II exemplifies a typical summary of the distribution of apigenin recovered from the *in vitro* absorption experiments presented in Figure 2 at a specific time point. Apigenin quantities were calculated from radioactivity data, the percentage of radioactivity as apigenin (Figure 3) and the specific activity of [G-^{3}H]-apigenin. Of the apigenin absorbed, a greater percentage was retained in SC with A/D (87% at 4 hr) compared with DMSO (65%) and PG (71%). In contrast, a greater percentage distributed into skin w/o SC with DMSO (25% at 4 hr) relative to A/D (9%), resulting in more apigenin absorption (140 ng vs. 65 ng of A/D). Although a higher percentage also distributed in skin w/o SC with PG/D (25%) relative to A/D, apigenin quantity was still lower (49 ng) as a result of much lower total absorption (2.9%).

At all the time points, more than 85% of the dose was recovered from the skin wash for these groups. The radioactivity in the surrounding skin was below 1% of the dose and not different among the three groups ($p = 0.96$ by Anova). Total drug recovery was quantitative for all groups (100.5 ± 0.5%).

Table III shows apigenin distribution in dermis and epidermis determined by epidermal scrapping in parallel *in vitro* absorption experiments with those summarized in Table II. *In vitro* apigenin distribution in epidermis was in the order of A/D > DMSO ($p < 0.02$ by *t*-Test) while the distribution in dermis was not different between A/D and DMSO ($p > 0.4$). Apigenin distribution was the lowest with PG/D in both epidermis and dermis.

Apigenin in viable epidermis, calculated from apigenin in dermis (Table III) and apigenin in skin w/o SC (viable epidermis plus dermis) (Table II), was in the order of DMSO (96 ± 23 ng) > A/D (23 ± 7 ng) [$p < 0.02$ by *t*-Test], DMSO > PG/D (31 ± 0.4 ng) [$p < 0.01$].

HPLC-Scintillation Analysis of *In Vitro* Samples

All the samples from *in vitro* absorption of [G-^{3}H]-apigenin for 7 hr exhibited a single radioactive peak in their HPLC-scintillation

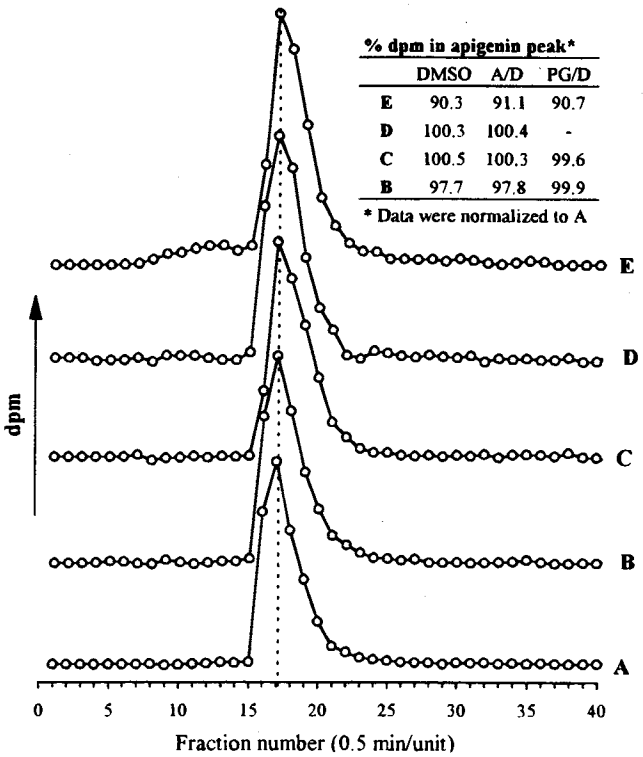

Fig. 3. HPLC-scintillation profiles of samples from *in vitro* percutaneous absorption of apigenin solution spiked with [G-^{3}H]-apigenin (10 μl, 25 mM, 16.7 μCi/μmole) for 7 hr. A. [G-^{3}H]-apigenin standard. B. epidermal extract. C. dermal extract. D. skin wash. E. receptor fluid.

profiles (Figure 3). Normalized to the [G-^{3}H]-apigenin standard, 100% of radioactivity was found in the apigenin peak for all the samples except for those from receptor fluid. About 90% of radioactivity in receptor fluid was in the apigenin peak and the other 10% distributed widely in the background. The HPLC-scintillation profiles were not different among the three vehicle groups.

DISCUSSION

Flavonoids have been extensively studied for a variety of pharmacological applications including chemotherapy and chemoprevention. However, little was known about percutaneous absorption of this important class of natural compounds. Our studies of apigenin percutaneous absorption focused on 6–7 hours to replicate the time period from drug treatment to ODC assay in previous chemoprevention studies (1).

The vehicle effects on skin tumorigenesis and ODC induction might be the result of differential drug delivery to the target site in the skin tissue (1). To test this hypothesis, apigenin uptake into epidermal cells in two vehicles was studied and found to be in an order (A/D > DMSO) opposite to that of chemoprevention (1,2). Apigenin distribution in epidermal tissue was also in this order shown both *in vivo* and *in vitro* (Table III).

Due to the low drug recovery of the *in vivo* absorption measurements, our further absorption studies employed the quantitative *in vitro* absorption model. Preliminary results showed that DMSO delivered more apigenin into receptor fluid through mouse skin, suggesting that DMSO could deliver more apigenin into deeper layers of skin relative to A/D. This hypothesis was proven by the studies of apigenin subtissue distribution within epidermis by tape-stripping and assessment of apigenin in epidermis and dermis. DMSO was found to deliver more apigenin into viable epidermis compared with A/D. This is consistent with the better chemopreventive activity associated with apigenin in DMSO. Thus, viable epidermis appears to be the target site of apigenin's chemoprevention. In addition, the skin cancer we examined previously (squamous cell carcinoma) originates in the viable epidermis (5), and ODC activity was reported to be primarily in viable epidermis (6).

In contrast, apigenin in A/D was mainly absorbed and deposited in SC. As the depth of skin increased, apigenin distribution decreased sharply to the same level, and finally became less in viable epidermis compared with DMSO. This pattern is clearly shown with the combination of radioactivity distribution in SC (Figure 1B) and skin w/o SC (Figure 2B). This is consistent with the reservoir function of SC reported with many hydrophobic drugs such as glucocorticoids (7).

The low absorption of apigenin in PG/D during the initial hours might contribute to the very low ODC inhibition associated with this vehicle (1). Our previous studies showed that apigenin pretreatment exerted a greater inhibition on TPA-induced ODC than apigenin treatment after TPA (unpublished data). This suggests that the inhibition of ODC is more important at the early phase of induction. However, both the rate and extent of apigenin percutaneous absorption with PG/D was very low in the initial hours although the absorption reached the same level with A/D by 4 hr (Figure 2). It is possible that the apigenin in PG/D may inhibit of TPA-induced ODC to a similar extent as apigenin in A/D or DMSO if it is applied 4–6 hr prior to TPA.

Our HPLC-scintillation data did not provide evidence of apigenin metabolism in mouse skin during *in vitro* percutaneous absorption (Figure 3). In receptor fluid, the 10% of radioactivity that did not associate with apigenin seemed to be multiple trace decomposed species. This is consistent with our previous *in vivo* metabolism studies (1).

The differences in percutaneous absorption among the three vehicles is likely to be the consequence of their different physicochemical properties. DMSO is known to enhance the skin permeability of other compounds by impairing the barrier function of SC, the main barrier for topical drug delivery (8). The characteristics of apigenin percutaneous absorption in DMSO were consistent with this property. Although A/D and PG/D also contained DMSO, the lower quantity caused less SC impairment that was proportional to DMSO quantity in a solvent mixture (8).

Compared with DMSO, acetone has higher volatility that kept apigenin in solution for shorter time, and lower penetration enhancing ability that limited the quantity of apigenin delivered into deeper layers of skin. In our studies, the solvent disappeared within 0.5 hr after topical application of apigenin in A/D, and apigenin precipitation was observed, while the solvent disappeared more than 2 hr after application in DMSO.

Because of the very low volatility of propylene glycol, the solvent could be seen during the entire absorption period. Thus, drug concentration in PG/D was lower than that in A/D or DMSO during absorption, and the release of apigenin to skin from the vehicle might be limited.

In summary, the vehicle (DMSO), in which apigenin showed better chemopreventive activity, delivered more apigenin to viable epidermis. Thus, delivering apigenin into viable epidermis appears to be a necessary property for an apigenin topical formulation to be effective in skin cancer prevention.

Apigenin concentrations in epidermal cells determined *in vivo* are in the same range as those we determined previously in cultured C50 keratinocytes (9). Apigenin in this range arrested cell cycle at G_2/M phase (9). Thus, these drug uptake data established a connection between the *in vivo* chemopreventive studies and the *in vitro* mechanistic studies.

Table III. Distribution of Apigenin in Mouse Skin After *In Vitro* Percutaneous Absorption for 4 hr[a]

	Apigenin (ng)		
Skin Section	DMSO	A/D	PG/D
Epidermis	451 ± 86	688 ± 120	151 ± 44
dermis	44 ± 18	42 ± 15	18 ± 9
Recovery (%)	98.9 ± 0.5	100.5 ± 0.4	99.5 ± 0.8

[a] The mouse skin w/o subcutaneous fat mounted on Franz cells was treated with apigenin solutions spiked with [G-^{3}H]-apigenin (10 μl, 25 mM, 16.7 μCi/μmole). Apigenin quantities are calculated from radioactivity data and expressed as Mean ± SE (n = 3). Recovery was calculated from the sum of apigenin recovered from skin sections and skin wash.

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