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THE IMPACT OF ETHANOL ON THE IN VITRO SKIN PENETRATION RATES OF CAFFEINE IN ENGINEERED SKIN CONSTRUCTS.

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ABSTRACT

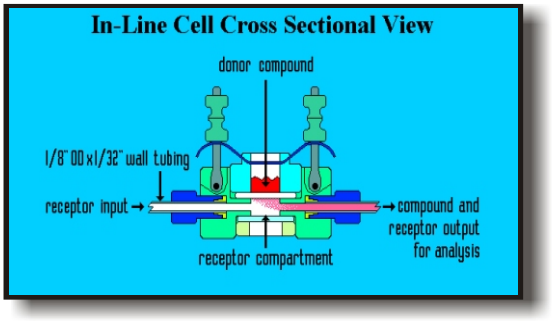
The reference material, caffeine (prepared in ethanol), was evaluated in 3 *in vitro* models to compare the rates of skin penetration in each of the models. The models were human donor skin, an engineered skin construct (MatTek Corporation, Model EPI-606X) and slaughterhouse-derived pig skin. The tissues were mounted in flow-through diffusion cells (PermeGear, Inc., 0.64 cm^2 surface area), qualified for barrier function by $^3H_20$ passage, followed by the application of a 9 µL dose of ^{14}C-caffeine in ethanol (~4 g/cm^2). The study duration was 24 hours. The recovery of caffeine was acceptable (typically 95 to 100%) in each model. In human and pig skin, the rates of skin penetration were uniform and continuous throughout the 24-hour period. The mean amounts of caffeine that had been absorbed after 24 hours were 11% and 15% of applied dose, respectively (n=2 trials). However, in the engineered skin model, the rate of penetration was remarkably high in the first 3 hours, with an abrupt decrease in the penetration rate thereafter. The amount absorbed after 3 hours was approximately 60% of applied dose. After 24 hours, the amount had increased to only 62% of applied dose. Subsequent experiments were conducted to evaluate the impact of the ethanolic vehicle on caffeine's penetration rate in engineered skin. In the first experiment, caffeine prepared in water was tested in parallel to caffeine prepared in ethanol. A notable penetration rate lag phase was observed in the water-based preparation as compared to the ethanol-based preparation, but totals absorbed were 87% vs. 66% of the applied dose, respectively. In the second experiment, engineered skin was pre-treated with ethanol followed by topical application of caffeine. Caffeine penetration rates and total caffeine absorption were similar in ethanol pre-treated and non-treated engineered skin. These results suggest that ethanol may have enhanced skin penetration upon initial exposure, but the solvent effect may have been rapidly modulated.

INTRODUCTION

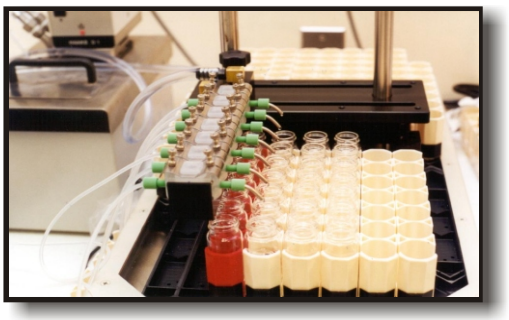
It is well established that percutaneous absorption is influenced by the vehicle containing the chemical. Therefore, the vehicle of choice plays a critical role in determining how much of the chemical is available systemically. Some vehicles hydrate the *stratum corneum* through occlusion, leading to enhanced dermal penetration, whereas others alter the integrity of the *stratum corneum* by interacting with membrane lipids. In addition, vehicles may have differential effects on the solubility of the chemical and ultimately, the concentration of the chemical available for absorption.

In previous experiments, we have shown differences in the kinetics of ethanolic solutions of caffeine through engineered skin as compared to human or pig skin.Caffeine rapidly penetrated engineered skin whereas significantly lower levels of caffeine were present in human or pig skin when the ethanol vehicle was used. Based on these discrepancies, we have evaluated the influence of two vehicles (ethanol and water) on the dermal penetration kinetics of caffeine in bioengineered human skin.

Schematic View of Diffusion Cell



Overall View of Diffusion Cells and Collection Apparatus



MATERIALS AND METHODS

Skin Models

Human skin was obtained from the Transplant Resource Center of MD dermatomed to 200-300 µm. The skin was stored at -20ºC. Full-thickness pig skin was obtained from a commercial supplier. The skin was clipped of bristles and dermatomed to 350-500 µm, and stored as above. Engineered human skin (Model Epi-606X™, MatTek Corp., Ashland, MA) consists of normal human epidermal keratinocytes cultured in 30 mm Millicell® inserts to form a highly differentiated, 3-dimensional construct, complete with basal, spinous, granular, and cornified layers. The MatTek Model Epi-606X™ was received on shipping agar, and used on the day of receipt.

Reference Material

- ^{14}C-Caffeine prepared as an ethanolic solution of ~280 µg/mL

Preparation of Skin Models

Human or pig skin was removed from the freezer and allowed to thaw at room temperature. The skin was examined for physical defects, and the thickness of the skin was measured. Squares of skin 2 to 4 cm^2 were cut from the sheet and mounted *stratum corneum*-side up into the diffusion cells. The engineered human skin was prepared by removing the culture inserts from the shipping agar and punching out 3/4" diameter tissue samples from the inserts.

- Diffusion cells: PermeGear flow-through diffusion cells (0.64 cm^2)
- Skin temperature: 32ºC, maintained by water-jacketed warmer
- Skin was equilibrated at least 30 minutes prior to assay

^3H_2O Skin Barrier Integrity Test

The skin barrier integrity test was conducted by applying 200 µL of ^3H_2O (~0.6 µCi) onto each tissue for 20 minutes. After 20 minutes, non-absorbed ^3H_2O was gently blotted from each tissue, and the tissues were maintained in the diffusion cells for an additional 60 minutes. The amount of ^3H_2O absorbed into the receptor fluid during the 80-minute test was determined by scintillation counting.

Standard Methods Used for the Initial Reference Materials Testing

The reference material caffeine was applied topically to each tissue. Total Activity Controls (to determine the total ^{14}C activity in each test dose) were prepared.

In Vitro Percutaneous Penetration Assay Design

- receptor fluids
  - water-soluble test materials:
    a modification of Hanks' Balanced Salt Solution (HBSS) w/o phenol red, w/o sodium bicarbonate, containing 25 mM HEPES, 1 g/L glucose, 7 g/L NaCl, and 100 µg/mL gentamicin
- receptor fluid flow rate: 25 µL/minute; 1.5 mL/hour
- reference material dose rate: finite dose of 4 µg/cm^2
- dose volume: 14 µL/cm^2
- duration of exposure: 24 hours
- receptor fluid fraction interval: each fraction had a duration of 6 hours in human skin; 3 hours in pig skin and in engineered skin
- number of fractions collected: 8

Caffeine exposures were terminated after 24 hours by washing and rinsing the non-absorbed test material from the tissues. The tissues were washed with 10% dish detergent and rinsed with deionized water. The wash and rinse solutions were blotted from each tissue with cotton swabs, and collected for subsequent scintillation counting. Each diffusion cell was drained of residual receptor fluid (Line fraction), and disassembled to remove the tissue. The epidermal side of each tissue was tape stripped to remove the *stratum corneum* for subsequent scintillation counting. Each of the *ex vivo* human or pig skins were tape stripped with a total of 10 strips of tape. The engineered human skin was tape stripped only one time to avoid damaging the tissue.

The epidermis and dermis of the *ex vivo* human and pig skins were separated following immersion in a 60ºC water bath for 1.5 minutes. All of the skin samples were digested overnight at 60ºC in Scintigest (Fisher Scientific). The digested samples were acidified to eliminate alkaline-mediated chemi-luminescence.

Scintillation Counting

All samples and total activity controls were scintillated with ReadySafe scintillation cocktail (Beckman), and the ^{3}H or ^{14}C activities determined using a Beckman LS6500 liquid scintillation counter.

Presentation of Data

The data are presented as "% of applied dose" using the following equation:

$$\%\ \text{of Applied Dose} = (^{14}\text{C absorbed} / {}^{14}\text{C total activity control}) \times 100$$

where "^{14}C absorbed" represents the amount of ^{14}C-caffeine detected in each of the receptor fluid fractions and skin compartments. Total absorption is defined as the total amount of material collected in the *stratum corneum*, epidermis, dermis and receptor fluid samples.

Subsequent Experiments to Evaluate the Impact of Ethanol on Caffeine Penetration in Engineered Skin

Comparison of Caffeine Prepared in Ethanol and Caffeine Prepared in Water

To determine the impact of the vehicle on caffeine penetration, ^{14}C-caffeine prepared in ethanol [^{14}C-caffeine (EtOH)] and ^{14}C-caffeine prepared in water [^{14}C-caffeine (H_2O)] were tested in parallel using essentially the methods described above.

- ^{14}C-caffeine (EtOH) was applied at a dose of 14 µL/cm^2 (4 µg/cm^2)
- ^{14}C-caffeine (H_2O) was applied at a dose of 42 µL/cm^2 (12 µg/cm^2)
  - The 3-fold greater dose volume was required to adequately cover the hydrophobic skin surface with the aqueous vehicle.
- receptor fluid fraction interval: The first 3 fractions had 1-hour durations;subsequent fractions had 3-hour durations.

Application of Ethanol 4 Hours Post ^{14}C-Caffeine Treatment

A 14 µL/cm^2 dose volume of ethanol was applied to a set of tissues 4 hours after the application of a standard dose of ^{14}C-caffeine (EtOH) to determine the impact of the ethanolic vehicle on the further penetration of caffeine. A set of control tissues treated only with ^{14}C-caffeine (EtOH) was tested in parallel. The methods used were essentially the same as described above with the following exceptions:

- receptor fluid fraction interval: The first 6 fractions had 1-hour durations; subsequent fractions had 3-hour durations.
- At the end of the 4th hour (at the end of fraction 4), a standard 14 µL/cm^2 dose volume of ethanol was applied to the test tissues.

Evaluation of Tissue Pretreated with the Ethanolic Vehicle

A standard 14 µL/cm^2 dose volume of ethanol was applied to a set of tissues 1 hour prior to the application of ^{14}C-caffeine (EtOH) to determine the impact of ethanol on the tissue. ^{14}C-caffeine (EtOH) was applied to untreated tissues in parallel. The methods used were essentially the same as described above with the following exception:

- receptor fluid fraction interval: The first 12 fractions had 20-minute durations; subsequent fractions had 3-hour durations.

ACKNOWLEDGEMENTS

The authors wish to thank MatTek Corporation for providing the engineered human skin for a number of the experiments. The authors also gratefully acknowledge the expert consultation of Dr. Robert L. Bronaugh and Dr. Jeff Yourick of the US Food and Drug Administration.

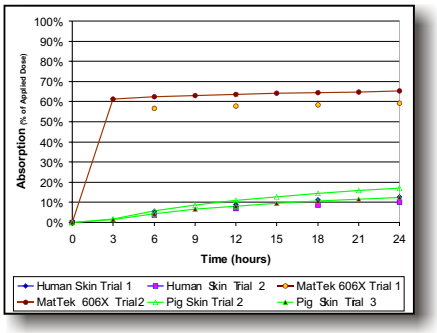


Figure 1. Percutaneous penetration of caffeine in three skin models (cumulative % of applied dose).

Model	^3H_2O passage % of applied dose (mean ± 1 s.d.)
human skin	0.223 ± 0.020 0.320 ± 0.026
pig skin	0.422 ± 0.122 0.400 ± 0.051
engineered human skin	1.614 ± 0.063 1.949 ± 0.469

Table 1. Skin Barrier Function Test Results

RESULTS OF THE INITIAL REF

Figure 1 presents the cumulative penetration curves and the total absorpti
rate curves are presented in Figure 2. The skin barrier test results for each
values for caffeine after 24 hours exposure are presented in Table 2. Reco

Human Skin

Since caffeine receptor fluid fractions were collected at 6-hour intervals, ra
achieved earlier than 6 hours. However, based upon the cumulative % of
uniform rate of penetration throughout the 24-hour period.

Pig Skin

Although total absorption of caffeine in pig skin was slightly higher than in
the skin penetration rate curve were very similar to those of human skin, in
period.

Model EPI-606X™

In contrast to the human and pig skin, the penetration rates in the enginee
caffeine penetration was remarkably high within the time that the first fract
with an abrupt decrease in the penetration rate thereafter. Since most of t
peak penetration rates may have been achieved in less than 3 hours after

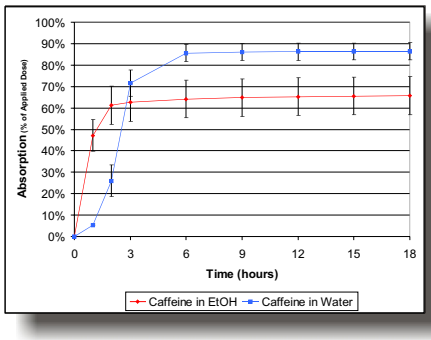


Figure 3. Percutaneous penetration of caffeine prepared in ethanol and in water (cumulative % of applied dose).

RESULTS OF THE SUBSEQUENT EXPER ETHANOL ON CAFFEINE PENE

Comparison of Caffeine Prepared in Ethanol and Caffeine Prepared in

Figures 3 and 4 present the cumulative penetration curves and the penetra
absorption of ^{14}C-caffeine (EtOH) was similar to the results reported above
hour in the first 3-hour period reveals a remarkably high penetration rate in
penetration rates by the third hour. In contrast, caffeine (H_2O) shows an in
hours. Interestingly, a significantly greater portion of the applied dose of c

RESULTS

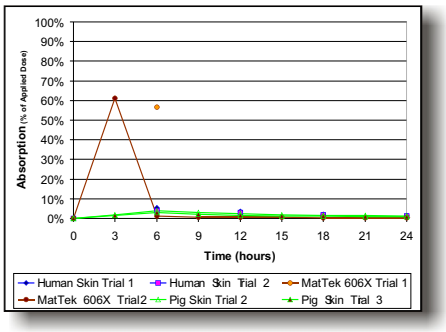


Figure 2. Percutaneous penetration of caffeine in three skin models (% of applied dose/fraction).

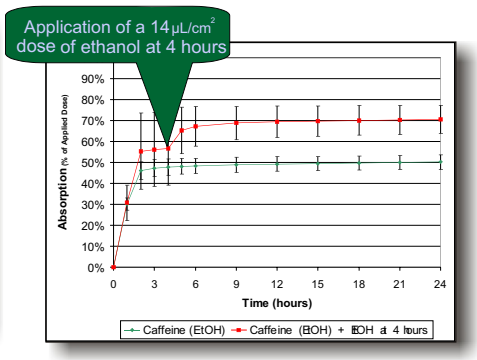


Figure 5. Application of ethanol 4 hours after caffeine treatment (cumulative % of applied dose).

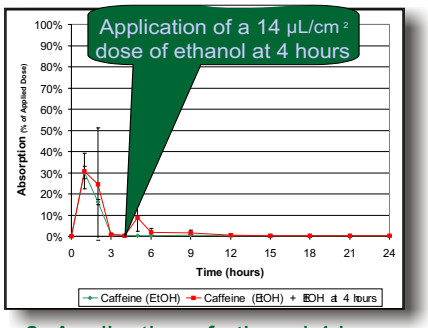


Figure 6. Application of ethanol 4 hours after caffeine treatment (% of applied dose/fraction).

Model	Caffeine (in ethanol) % of applied dose
human skin	14.0 ± 4.4 11.4 ± 4.1
pig skin	24.4 ± 6.0 17.3 ± 2.9
engineered human skin	60.3 ± 7.1 66.3 ± 7.6

Data (mean ± 1 s.d.) of two independent trials per test group include total absorption into receptor fluids, *stratum corneum*, and epidermal/dermal compartments.

Table 2. Total Skin Absorption at 24 Hours (% of Applied Dose)

ERENCE MATERIALS STUDIES

on of caffeine at 24 hours in each of the 3 skin models. The penetration
n of the 3 skin models are summarized in Table 1. The total absorption
very of radiolabeled caffeine exceeded 90% in all three models.

ther than at 3-hour intervals, peak penetration rates may have been
applied dose and skin penetration rate curves, there appears to be a fairly

human skin, the profiles of the cumulative % of applied dose curve and
ndicating a relatively uniform rate of penetration throughout the 24-hour

red model were not uniform throughout the 24-hour period. For example,
ion was collected (6 hours and 3 hours, for trials 1 and 2, respectively)
he penetration of caffeine had occurred within the first collected fraction,
caffeine application.

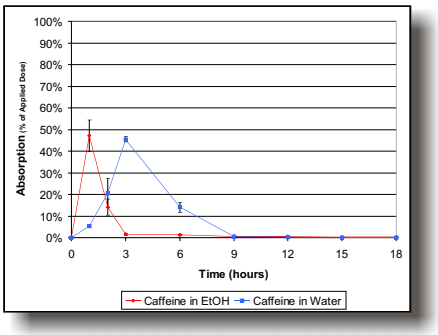


Figure 4. Percutaneous penetration of caffeine prepared in ethanol and in water (% of applied dose/fraction).

RIMENTS TO EVALUATE THE IMPACT OF TRATION IN ENGINEERED SKIN

n Water

ation rate curves for caffeine prepared in ethanol and in water. The total
e. However, the additional resolution obtained by collecting fractions every
n the first hour after application, followed by the rapid decrease in
itial lag phase in the first hour, with peak penetration rates achieved at 3
affeine (H_2O) penetrated the engineered skin relative to caffeine (EtOH).

Application of Ethanol 4 Hours Post ^{14}C-Caffeine Treatment

The cumulative ^{14}C-caffeine (EtOH) penetration curve and the penetration rate curve for tissues treated with ethanol 4 hours after caffeine treatment are presented in Figures 5 and 6, respectively. The results of caffeine (EtOH) tested in parallel as a control are presented in Figures 5 and 6, as well. A significant increase in the penetration rate of caffeine was observed immediately after the application of the ethanol at the 4-hour time point. The enhancement of penetration was evident for at least 2 more hours before the penetration rates decreased to control levels. These results demonstrate that ethanol provides an enhancement of the penetration rate of caffeine, perhaps carrying caffeine through the tissue as a solvent.

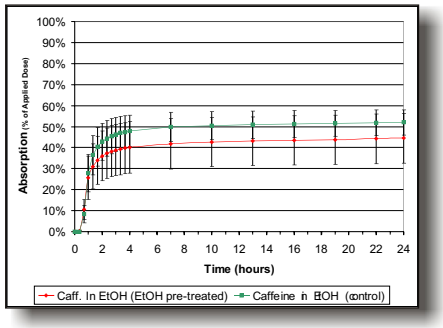


Figure 7. Percutaneous penetration of caffeine in tissue pretreated with ethanol (cumulative % of applied dose).

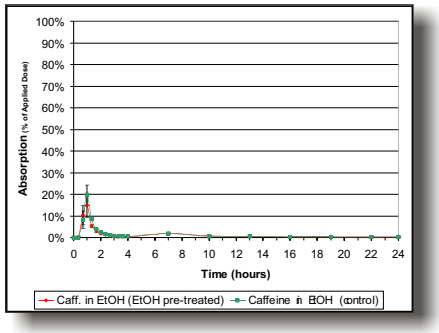


Figure 8. Percutaneous penetration of caffeine in tissue pretreated with ethanol (% of applied dose/fraction).

Evaluation of Tissue Pretreated with the Ethanolic Vehicle

The ^{14}C-Caffeine (EtOH) penetration curve and the penetration rate curve for tissues pretreated with ethanol are presented in Figures 7 and 8, respectively. The penetration rates of caffeine (EtOH) tested in pretreated or control tissues do not appear to differ significantly in this experiment, although the total penetration in the ethanol pretreated tissues does appear to be less than results typically obtained from untreated tissues under standard conditions. The results may suggest that a secondary effect of the ethanolic vehicle on the tissue may be an attenuation of the penetration of caffeine. Accordingly, further investigations of the optimum timeframe for application of the ethanol to obtain the greatest attenuation effect may be warranted.

CONCLUSIONS

The results suggest that ethanol behaves as a penetration enhancer upon initial application to the test system. Ethanol appears to be an effective carrier of caffeine as topically-applied ethanol penetrates the tissue model. However, the penetration enhancing effect of ethanol on the penetration of caffeine appears to be rapidly attenuated. This attenuation may occur as the ethanol evaporates or is dissipated into the model, or perhaps as ethanol changes the hydration state of the engineered tissue model. Understanding the differences in the engineered tissue relative to the *ex vivo* models may provide some insight into the basis of the different penetration kinetics observed for caffeine prepared in an ethanolic vehicle.

FORWARD ACTIONS

- Conduct additional experiments to determine the optimal timeframe for ethanol pretreatment of tissues to induce a significant attenuation of caffeine penetration.
- Conduct experiments to understand changes in tissue hydration after treatment with different vehicles.

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