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Research Article

Percutaneous Penetration Enhancement *in Vivo* Measured by Attenuated Total Reflectance Infrared Spectroscopy

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A novel application of attenuated total reflectance IR spectroscopy (ATR-IR) was used to monitor the outer several microns of the stratum corneum (SC) and, thereby, demonstrate enhanced percutaneous absorption *in vivo* in man. 4-Cyanophenol (CP) as a model permeant yielded a unique IR signal, distinct from those of the stratum corneum and the vehicle components. CP was administered for 1, 2, or 3 hr as a 10% (w/v) solution either in propylene glycol or in propylene glycol containing 5% (v/v) oleic acid. The absorbance at 2230 cm^{-1}, which corresponded to C≡N bond stretching, diminished significantly faster when CP was codelivered with oleic acid. An IR absorbance due primarily to propylene glycol at 1040 cm^{-1} (C–O stretching) also disappeared more quickly following application of the enhancer-containing solution. In addition, only the formulations with oleic acid induced a higher wavenumber shift in the frequency of the asymmetric C–H bond stretching absorbance. This change indicates increased lipid-chain disorder, the mechanism by which oleic acid is believed to cause enhanced drug transport across the stratum corneum. Therefore, ATR-IR permits one to examine noninvasively the kinetics, extent, and mechanism of percutaneous penetration enhancement *in vivo* in human subjects.

KEY WORDS: skin penetration; transdermal enhancement; oleic acid; 4-cyanophenol; infrared spectroscopy.

INTRODUCTION

Because the skin is such an excellent barrier to topically administered drugs, strategies for the effective and reversible enhancement of transdermal penetration must be examined (1). The barrier function of skin's outermost layer, the stratum corneum (SC), is firmly established (2). The characterization of the SC as a wall (with the corneocyte "bricks" cemented by the intercellular, lamellar lipid "mortar") is an approximate but reasonable model (3), and passive molecular diffusion through the SC is thought to proceed via the intercellular domains (e.g., Refs. 4–8). It follows that these lipid lamellae represent one target for penetration enhancer action.

Microscopic and biophysical probing of SC lipid structure has revealed a well-ordered, lamellar organization (9,10). Results from studies using transmission infrared (IR) spectroscopy and differential scanning calorimetry showed that the lipid transitions occur at temperatures well above 37°C (11–13). Further, the phase transition temperatures were significantly lowered by SC pretreatment with putative penetration enhancers (e.g., *cis*-unsaturated fatty acids, such as oleic acid). At physiological temperature, Fourier transform (FT)-IR spectroscopic results indicated that certain enhancers increased SC lipid disorder. Since these same promoters enhanced drug flux across the skin, the hypothesis was proposed that the enhancement mechanism was lipid "fluidization" (12).

Recently, the deductions based upon the transmission IR studies of porcine SC have been confirmed for human SC *in vivo* (14). The action of oleic acid, specifically, on the SC of human volunteers was monitored using attenuated total reflection (ATR)-FTIR (15). Similar lipid disordering effects were noted and a sustained action of the fatty acid was apparent. It also proved possible to approximate the amount of oleic acid present within the outer layers of the SC, by assigning an absorbance at 1710 cm^{-1} to the carbonyl stretching of the fatty acid's carboxyl group. There was a reasonable correlation between the magnitude of this *in vivo* absorbance and the uptake of enhancer into excised SC *in vitro*, as a function of applied oleic acid concentration.

The latter finding suggests, in theory, that ATR-IR can be similarly used to assess the concentration of a topically administered drug in the outer region of the SC, provided that a unique IR absorbance, distinct from those of the SC, can be identified. While the SC does absorb radiation extensively in the mid-IR region, IR transparent "windows" exist, most notably between about 1700 and 2500 cm^{-1} and beyond about 3600 cm^{-1} (15). The SC also absorbs little radiation in the near-IR. Further, the ATR-IR method can also measure other constituents of the delivery system (co-

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solvent, enhancer, etc.) along with changes in SC lipid and protein absorbances induced by the application of the drug delivery system.

Thus, the aim of this study was to examine the potential of ATR-FTIR to measure percutaneous penetration enhancement *in vivo* in man. The penetrant used, 4-cyanophenol, was chosen for its IR signature, in particular the intense C≡N stretching absorbance at 2230 cm^{-1}. The specific objectives were (a) to follow the penetrant in the SC in the presence and absence of a penetration enhancer (oleic acid), (b) to investigate whether the solvent (propylene glycol) could also be monitored, and (c) to determine the action of the enhancer on the SC lipid domains.

MATERIALS AND METHODS

Attenuated Total Reflectance Infrared Spectroscopy in *Vivo*

The volunteers for these studies were five healthy adults (aged 25–40 years), who were receiving no medication and had no history of skin disease. For the purposes of the experiments described below, the subjects were dosed topically with either a 10% (w/v) solution of 4-cyanophenol (CP; Aldrich Chemical Co., Milwaukee, WI) in propylene glycol or the same CP solution containing 5% (v/v) oleic (*cis*-9-octadecenoic) acid (Sigma, St. Louis, MO). The selection of CP was based upon (a) its relatively efficient absorption across human skin (16) and (b) its IR spectral features (see above). The dosed and control sites were the midventral forearm, and both were wiped briefly with an ethanol-soaked Q-tip prior to the experiment. The formulations (500 μl) were delivered via a 3 × 8-cm^2 Secure gauze pad (Johnson & Johnson, New Brunswick, NJ), which was affixed to the skin with overlaying adhesive tape. The application time was 1, 2, or 3 hr. At the end of the treatment period, the gauze pad was removed, and the skin surface was wiped clean, gently and quickly, with another ethanol-soaked Q-tip. ATR-IR spectra were recorded (i) before treatment, (ii) about 60 sec following the cleaning posttreatment, and (iii) then serially over the next 9 hr. During the latter period, the volunteers pursued their normal activities, keeping the treatment areas unoccluded.

IR spectra of the subjects' forearms were measured using an Analect FX-6200 FTIR spectrophotometer (Laser Precision, Irvine, CA) equipped with an ATR sampling device (Skin Analyzer from Spectra-Tech, Stamford, CT). The ATR "analyzer" maintained the internal reflection element (IRE) parallel to the optical bench, allowing comfortable placement of the subject's arm onto the optics (14). The administration site was delineated with a felt-tip marker to ensure reproducible repositioning of the subjects' arms for each spectral measurement. Three trapezoidal IREs (approximate dimensions, 7 cm × 2 cm × 2 mm), which could be easily interchanged, were employed: ZnSe cut at an angle of 60°, ZnS cut at 60°, and Ge cut at 30°. These IREs permitted the IR beam to sample depths of between 0.8 μm (ZnSe) and 2 μm (Ge) into the stratum corneum (SC) (17). These beam penetration distances are not absolute; rather, the radiation within the tissue describes a decay function from which little useful information is obtained beyond the stated depths. Spectra were recorded with each IRE at each time point. Measurements were made at ambient laboratory conditions (relative humidity, 30–40%; temperature, 23 ± 1°C). The FTIR spectrophotometer enabled rapid acquisition and analysis of the spectra (15): each measurement represented an average of about 64 scans obtained over a 2-min period. To improve the digital resolution of the instrument (2.7 cm^{-1}), IR peak frequency maxima were determined using a center-of-gravity algorithm which allowed a precision of 0.1 cm^{-1} to be obtained (18).

Effect of Oleic Acid on the Uptake of CP into Stratum Corneum

In all skin penetration enhancement experiments, it is important to distinguish the true "kinetic" action of the enhancer from its "thermodynamic" effect on the SC/vehicle partitioning of the permeant (19). Therefore, we measured the uptake of CP into excised human SC from (i) a 10% (w/v) solution in propylene glycol and (ii) the identical solution containing 5% (v/v) oleic acid. Human SC, obtained from cadavers, was prepared as previously described (14) and was stored desiccated before use. For the uptake experiment, about 10 mg of SC was first rehydrated at 95% relative humidity overnight. The tissue was then weighed and incubated for 2 hr with 5 cm^3 of one of the two CP solutions identified above. The uptake of CP into SC was quantified radiochemically: the two solutions contained ^{14}C-labeled CP (Moravek Biochemicals, Brea, CA) to a final specific activity of 120 dpm/μg. At the end of the incubation period, the SC was removed from the CP solution, rinsed quickly in cold ethanol to remove surface chemical, dried on a wire mesh, and then subjected to liquid scintillation counting. Five replicates were performed at ambient laboratory temperature (23°C).

Effect of Oleic Acid on the *in Vitro* Skin Permeation of CP

For comparative purposes, the effect of the enhancer on the flux of CP across excised skin *in vitro* was measured. Fresh porcine skin, dermatomed to 350 μm, separated the donor and receiver chambers of a Franz-type diffusion cell (area, 1.8 cm^2) (12). The receptor solution was 5.5 ml of Dulbeccos' modified Eagle medium, supplemented with 5% fetal bovine serum, at pH 7.4. The donor solution was either (a) 250 μl of 10% (w/v) CP in propylene glycol, containing ^{14}C-CP, or (b) the identical solution containing 5% (v/v) oleic acid. Following application of the donor solution, which was open to the atmosphere, samples were removed periodically from the receiver chamber and were analyzed by liquid scintillation counting. When the amount of ^{14}C-CP appearing in the receptor solution per unit time became constant, the apparent steady-state flux of the permeant was calculated in the normal way (12). The experiments were performed in triplicate at ambient temperature (23°C).

RESULTS

Figure 1 shows representative IR spectra of (a) untreated human SC, (b) 4-cyanophenol (CP), and (c) human SC following application of a 10% (w/v) solution of CP in propylene glycol. The SC spectra in Fig. 1 were obtained *in vivo* using ATR-IR in conjunction with a ZnSe IRE. The

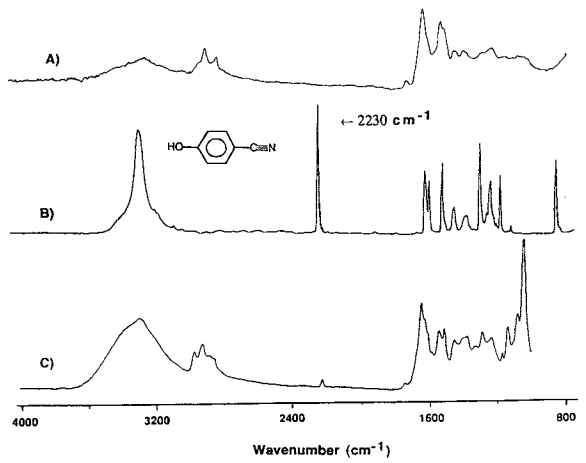

Fig. 1. Representative IR spectra of (A) untreated human SC, (B) 4-cyanophenol (CP), and (C) human SC following application of a 10% (w/v) solution of CP in propylene glycol.

intense C≡N stretching absorbance from CP at 2230 cm^{-1} is easily discernible in the spectrum of SC following treatment. Also visible is an intense absorbance (relative to the untreated SC spectrum) at 1040 cm^{-1}, which results primarily from the C–O stretching vibration of propylene glycol.

The absorbances at 2230 and 1040 cm^{-1} were used to determine the relative amounts of CP and propylene glycol, respectively, in the SC after removal of the delivery systems. To do so, these absorbances were normalized with respect to the SC absorbance at 1741 cm^{-1}. This peak originates from the C=O stretching vibration of endogenous SC lipids, and the normalization procedure eliminates intermeasurement differences due to variable degrees of skin contact with the IRE (14,15).

Figures 2A, 3A, and 4A show plots of the CP absorbance ratio (2230 to 1741 cm^{-1}) as a function of time postremoval of the formulations following 1, 2, and 3 hr of application, respectively. The effect of oleic acid in the vehicle may be observed from these graphs. In Figs. 2B, 3B, and 4B, a semilogarithmic transformation of the data is presented. The linearity of the resulting plots suggests an apparent first-order elimination of CP from the upper SC. Oleic acid impacts significantly on Figs. 2, 3, and 4 in two ways. (i) With increasing treatment time, it progressively increases the apparent first-order disappearance rate constant of CP from the SC. (ii) Concomitantly, immediately postremoval of the delivery system, it reduces the relative amount of CP observed spectroscopically (that is, the absorbance ratio in the first spectrum taken postremoval of the formulations containing oleic acid is less than that observed following treatment with the enhancer-free control; the difference increases with increasing treatment time). These effects of oleic acid are summarized in Table I.

Figures 5A–C show the propylene glycol absorbance ratios (1040 cm^{-1}/1741 cm^{-1}) plotted as a function of time postremoval of the formulations following 1, 2, and 3 hr of application, respectively. As for CP, oleic acid hastens the clearance of propylene glycol from the SC. However, a semilogarithmic transformation of the data in Fig. 5 does not lead to linearization (graphs not shown).

Oleic acid also caused a dramatic change in the peak

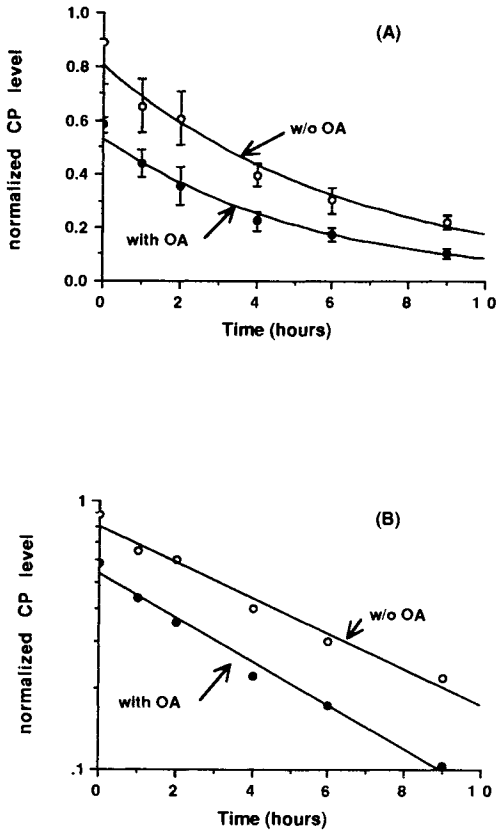

Fig. 2. Normalized CP level [expressed as (A) the 2230 cm^{-1}/1741 cm^{-1} IR absorbance ratio in the upper regions of human SC or (B) the log(absorbance ratio)] as a function of time following pretreatment for 1 hr with either a 10% (w/v) solution of CP in propylene glycol (open symbols) or the identical solution containing 5% (v/v) oleic acid (filled symbols). The data represent the mean ± SEM for 5 subjects.

frequency of the C–H asymmetric stretching vibration (2920 cm^{-1}), which results primarily from methylene groups in the SC lipid acyl chains (13). Figure 6 shows that all three treatments (1, 2, or 3 hr) with the oleic acid-containing formulations caused significant and sustained higher-wavenumber shifts in the absorbance, indicating increased lipid-chain disorder ("fluidization"). On the other hand, when CP was delivered in propylene glycol alone, without enhancer, no significant changes in the lipid absorbances were observed (Fig. 7). Parenthetically, and as we have reported previously (14), the presence of oleic acid in the SC could also be monitored by following the 1710 cm^{-1}/1741 cm^{-1} absorbance ratio.

Finally, with respect to the spectroscopy, it should be noted that the pattern of observations and the relative degree to which they occurred were similar for each of the IREs employed. The data presented represent all results, for the three IREs, averaged over the subject population. Any conclusions drawn from any one IRE are identical to those from any other. It appears, therefore, that the experiments report on events taking place throughout the outer few microns of the SC.

Table II indicates that 5% oleic acid caused a 35-fold increase in CP flux across porcine skin *in vitro*, while Table III suggests that the enhancer did not significantly affect

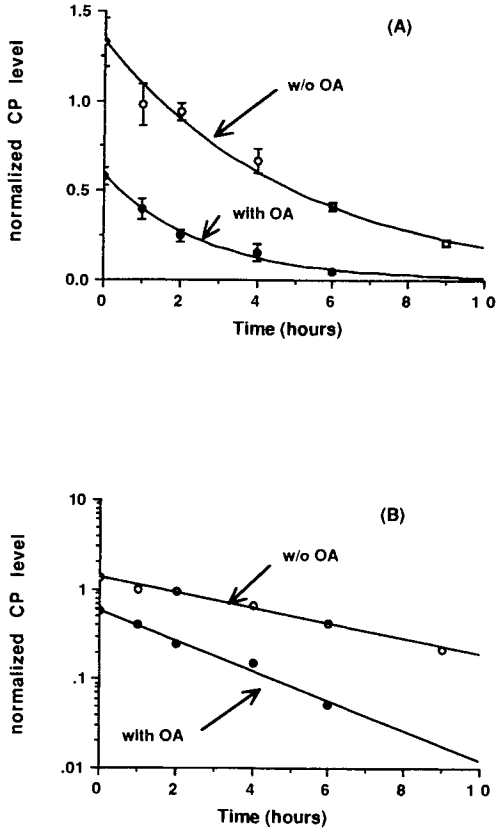

Fig. 3. Normalized CP level [expressed as (A) the 2230 cm^{-1}/1741 cm^{-1} IR absorbance ratio in the upper regions of human SC or (B) the log(absorbance ratio)] as a function of time following pretreatment for 2 hr with either a 10% (w/v) solution of CP in propylene glycol (open symbols) or the identical solution containing 5% (v/v) oleic acid (filled symbols). The data represent the mean ± SE for five subjects.

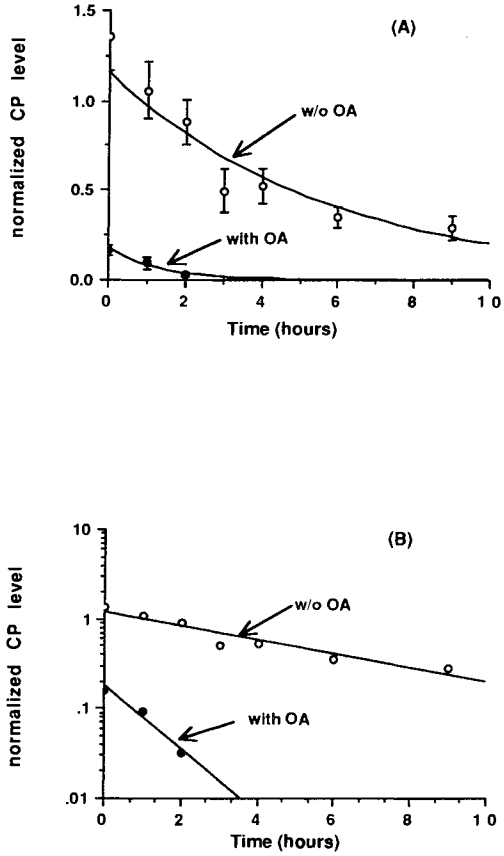

Fig. 4. Normalized CP level [expressed as (A) the 2230 cm^{-1}/1741 cm^{-1} IR absorbance ratio in the upper regions of human SC or (B) the log(absorbance ratio)] as a function of time following pretreatment for 3 hr with either a 10% (w/v) solution of CP in propylene glycol (open symbols) or the identical solution containing 5% (v/v) oleic acid (filled symbols). The data represent the mean ± SE for five subjects.

uptake of penetrant into the SC. Taken together, these results indicate that oleic acid is a true enhancer for CP and does not improve penetration by facilitating the partitioning of penetrant into the SC.

DISCUSSION

At an elementary level, the results reported meet the goals of the study: the ATR-IR technique has indeed allowed the *in vivo* percutaneous penetration enhancement of a model drug to be monitored. The solvent for the drug has also been followed spectroscopically, as has the effect of the enhancer on the SC. These qualitative deductions are now examined more closely in an initial attempt to quantify the kinetics, extent, and mechanism of the enhancement observed.

Figures 2, 3, and 4 show that, in the absence of oleic acid, as the treatment time increases, the relative amount of CP in the SC, when the first IR spectrum is obtained (i.e., at the end of the treatment period), reaches an apparent plateau. In the presence of enhancer, on the other hand, this initial, relative amount is always less and *decreases* with increasing treatment time (see Table I). The decay of the CP absorbance following removal of the delivery system is faster, however, following treatment with oleic acid (again, see Table I). We suggest that these observations are consistent with the hypothesis that, while CP is delivered into the SC at comparable rates from the formulations with and without enhancer, the presence of oleic acid promotes percutaneous transport and facilitates "throughput" of the penetrant. To illustrate this point, consider Fig. 8: the SC

Table I. Effect of 5% (v/v) Oleic Acid (OA) on (a–c) the Normalized CP Level (A_{sc}) Within the SC Compartment at the End of the Pretreatment Period and (d–f) the Apparent First-Order Elimination Rate Constant (k_e/hr^{-1}) of CP from the SC Following Delivery System Removal (n = 5; Mean ± SE)

	Pretreatment time (hr)		
	1	2	3
(a) A_{sc} w/o OA	0.82 ± 0.11	1.27 ± 0.12	1.22 ± 0.09
(b) A_{sc} with OA	0.56 ± 0.05	0.56 ± 0.04	0.15 ± 0.03*
(c) Ratio (a/b)	1.46	2.27	8.13
(d) 10 × k_e w/o OA	0.68 ± 0.06	0.91 ± 0.08	0.88 ± 0.06
(e) 10 × k_e with OA	1.17 ± 0.14	1.76 ± 0.19	2.44 ± 0.31*
(f) Ratio (e/d)	1.72	1.95	2.79

* Significantly different from 1-hr values; $P < 0.05$.

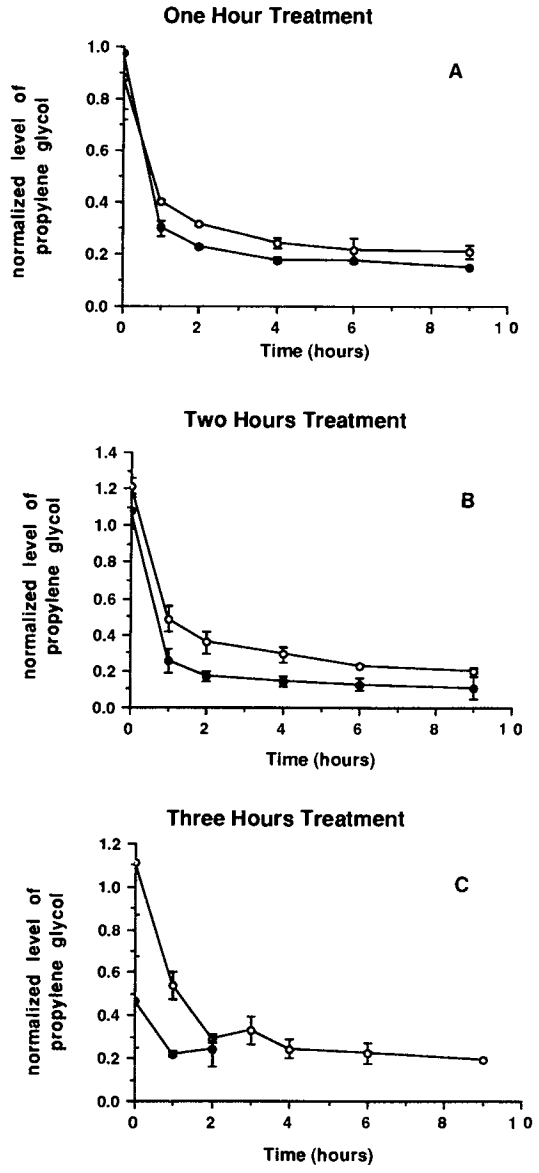

Fig. 5. Normalized level of propylene glycol (expressed as the 1040 cm^{-1}/1741 cm^{-1} IR absorbance ratio) in the upper regions of human SC as a function of time following pretreatment for (A) 1 hr, (B) 2 hr, and (C) 3 hr with either a 10% (w/v) solution of CP in propylene glycol (open symbols) or the identical solution containing 5% (v/v) oleic acid (filled symbols). The data represent the mean ± SE for five subjects.

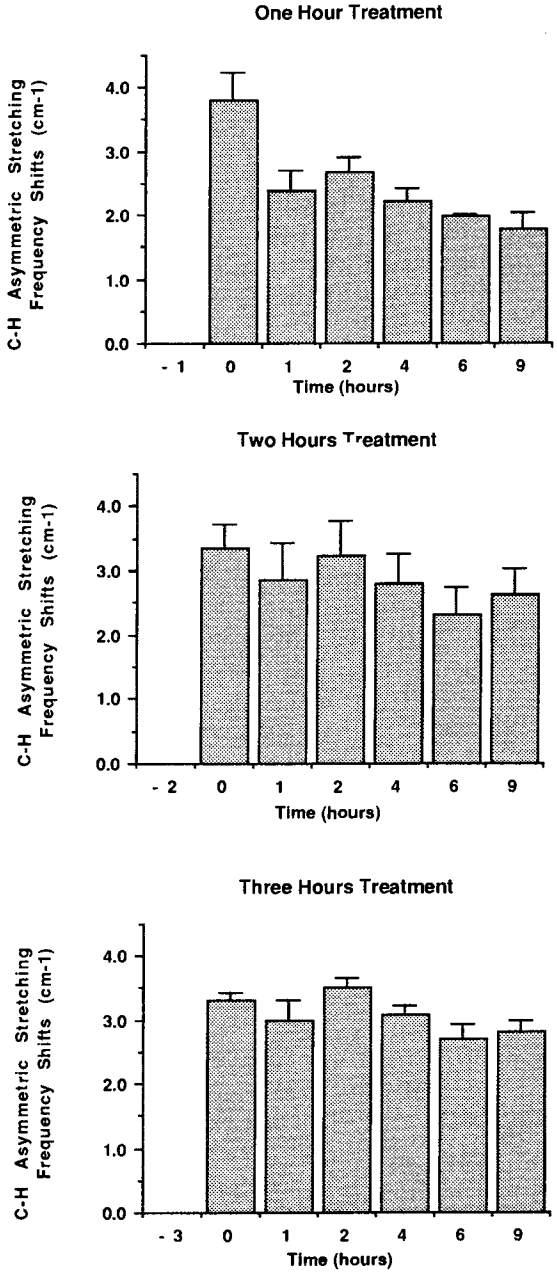

Fig. 6. Changes in the C–H asymmetric stretching frequency from human SC as a function of time following 1, 2, and 3 hr of treatment with a 10% (w/v) solution of CP in propylene glycol containing 5% (v/v) oleic acid. The zero changes indicated at −1, −2, and −3 hr represent the pretreatment controls. The data are the mean ± SE for five subjects.

"compartment" is that part of the membrane "seen" by ATR-IR. In these experiments, this represents the upper region of the SC. Assume that CP (a) is delivered into this compartment with zero-order kinetics (k_o/μg hr^{-1}) and (b) is cleared with first-order kinetics (k_e/hr^{-1}). The instantaneous amount of CP in the SC (A_{sc}/μg) can then be determined by solving the simple differential equation

$$dA_{sc}/dt = k_o - k_e \cdot A_{sc} \quad (1)$$

The result, of course, is

$$A_{sc} = (k_o/k_e)\,(1 - e^{-k_e t}) \quad (2)$$

Furthermore, at steady state

$$A_{sc} \rightarrow k_o/k_e \quad (3)$$

It follows that, if k_o remains relatively constant but k_e is increased, then A_{sc} must *decrease*. As an analogy, consider the effect of increasing clearance on drug steady-state plasma concentration (C_{ss}) while maintaining a constant iv infusion rate: C_{ss} would decrease, and, if the infusion were then stopped, plasma levels would decay more rapidly. To what extent do the data presented support this theory? Certainly, the decay of the CP signal postremoval of the delivery systems supports an apparent first-order elimination process

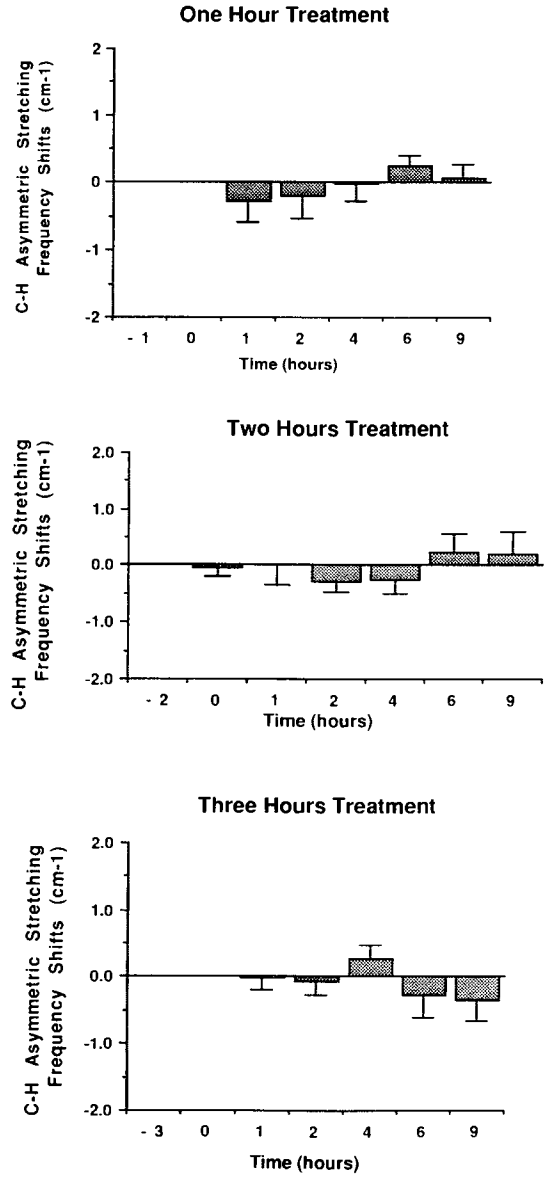

Fig. 7. Changes in the C–H asymmetric stretching frequency from human SC as a function of time following 1, 2, and 3 hr of treatment with a 10% (w/v) solution of CP in propylene glycol. The zero changes indicated at −1, −2, and −3 hr represent the pretreatment controls. The data are the mean ± SE for five subjects.

from the SC compartment. The zero-order input of CP cannot be verified; nevertheless, there is an excess of penetrant in the delivery system, and in the *in vitro* experiments, at least, a steady-state flux is achieved quite rapidly. The uptake of CP into human SC (Table III) was not changed significantly ($P > 0.05$) by the presence of 5% (v/v) oleic acid in the vehicle. Table I reveals a lower A_{sc} when the enhancer is used, opposite to the effect predicted solely on the basis of increased k_o. Thus, the possible change in k_o (due, for example, to altered penetrant partitioning) appears to be a secondary effect relative to the oleic acid-enhanced clearance of CP from the SC. [One should recognize, however, that these deductions are based upon experiments (*in vivo* and *in vitro*) which, although complementary, have not been performed

Table II. Effect of 5% (v/v) Oleic Acid on the *in Vitro* Steady-State Flux of 4-Cyanophenol Across Full-Thickness Porcine Skin ($n = 3$; Mean ± SD)

Vehicle	Flux ($mg \cdot hr^{-1} \cdot cm^{-2}$)
(A) Propylene glycol	1.82 ± 0.84*
(B) Propylene glycol containing 5% (v/v) oleic acid	53.6 ± 15.1*
Enhancement factor (B/A)	35

* These values are significantly different at $P < 0.01$.

under identical conditions.] The simplistic theory above, furthermore, predicts that A_{sc} and k_e are inversely related; the ratios calculated in Table I, therefore, should be equal. The fact that they are not reflects the elementary nature of the model, the rather severe assumptions, and the fact that the observations used to determine the k_e parameters are made after removal of the occlusive delivery system (during which time the SC will be undergoing additional changes as its hydration state returns to normal). Despite all these caveats, though, the basic hypotheses are reasonably well supported. (i) Oleic acid causes more rapid clearance of CP from the SC compartment measured. (ii) Input rate of CP is not dramatically affected by oleic acid in the vehicle; hence, because of the increased clearance, the level of CP initially measured in the SC at the end of the treatment periods is lower when oleic acid is present.

The quantitation of this work needs a firmer base. Specifically, the CP distribution and level throughout the SC must be determined, as a function of time and treatment. The propylene glycol data (Fig. 5) also require further examination. The clearance of the solvent from the SC appears to be biexponential, with a very rapid initial phase. Oleic acid enhances this "throughput" process too, which suggests a possible synergy of effect between enhancer and solvent. The literature contains a number of examples of such cooperativity (20).

The disordering of the SC lipid domains caused by oleic acid (Fig. 6) agrees with previously published work (12,14), in which the enhancer was delivered as an ethanolic solution. As before, a rapid onset of action, sustained over a prolonged period postremoval of the formulation, is observed. The effect is saturable (i.e., the lipid disordering does not continually increase) but is prolonged as the exposure to the enhancer is extended. The higher-wavenumber shift of the C-H asymmetric stretching vibration is associated with an increase in the number of "gauche" conformers

Table III. Effect of 5% (v/v) Oleic Acid on the *in Vitro* Uptake of 4-Cyanophenol into Excised Human SC from Two Vehicles ($n = 5$; Mean ± SE)

Vehicle	Uptake of 4-cyanophenol (μg/mg SC)
Propylene glycol	74.7 ± 9.7*
Propylene glycol containing 5% oleic acid	140.0 ± 31.5*

* These values are not significantly different at $P < 0.05$.

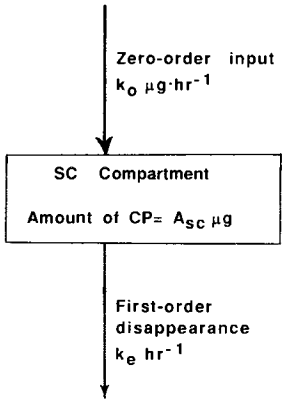

Fig. 8. Simple kinetic model used for the analysis of the results in Figs. 2–4 and in Table I. The SC "compartment" is that part of the membrane which can be "seen" by the ATR-IR spectrophotometer.

along the lipid hydrocarbon chains (21). By way of comparison, note that the melting of a pure hydrocarbon, such as hexadecane, causes a shift of about 10 cm^{-1}; it follows that the impact of oleic acid on the SC intercellular lipid domains is significantly disruptive. Thus, it would appear that the SC barrier is compromised by oleic acid for periods significantly longer than the contact of delivery system with skin. This seems reasonable given the favorable uptake of oleic acid by human SC (14) and its likely reluctance, therefore, to partition out of this lipophilic skin layer into the more aqueous underlying tissue.

In summary, this paper presents a novel, noninvasive examination of percutaneous penetration enhancement *in vivo* in man. The ATR-IR spectroscopic technique has permitted the effect of the enhancer on both drug and cosolvent flux in the SC to be determined and the mechanism of action of oleic acid to be considered.

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