Validation of Reflectance Infrared Spectroscopy as a Quantitative Method to Measure Percutaneous Absorption *In Vivo*

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Attenuated total-reflectance infrared (ATR-IR) spectroscopy has been used to follow the penetration of a model compound (4-cyanophenol; CP) across human stratum corneum (SC) *in vivo*, in man. CP was administered for periods of 1, 2, or 3 hr, either (a) as a 10% (w/v) solution in propylene glycol or (b) in an identical vehicle which also contained 5% (v/v) oleic (*cis*-9-octadecenoic) acid. At the end of the treatment periods, SC at the application site was progressively removed by adhesive tape-stripping. Prior to the removal of the first tape-strip, and after each subsequent tape-strip, an ATR-IR spectrum of the treated site was recorded. The presence of CP, as a function of position in the SC, was monitored spectroscopically via the intense C≡N stretching absorbance at 2230 cm^{-1}. The absolute amount of CP, as a function of SC depth, was determined by "spiking" the applied solutions with ^{14}C-labeled compound and subsequent liquid scintillation counting of the removed tape-strips. The presence of oleic acid in the applied formulation significantly increased the rate and extent of CP delivery as evaluated by either spectroscopy or radiochemical analysis. Furthermore, the ATR-IR and direct ^{14}C analysis of CP as a function of SC position were highly correlated. These data strongly support, therefore, the validation of ATR-IR as a quantitative tool to assess percutaneous penetration *in vivo*.

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

INTRODUCTION

The local and systemic bioavailability of topically applied drugs depends upon the kinetics and extent of the percutaneous absorption process. The definition of "bioavailability," in the context of dermatotherapeutics, has raised several issues and highlighted limitations of currently employed techniques (1). Ultimately, the aim of topical drug administration to diseased skin is the induction of a local therapeutic response by achieving an appropriate drug level at the affected site. Accurate bioavailability evaluation in this instance, demands measurement of drug levels at or near the site of action, i.e., within the skin tissue. Measurements in compartments other than the skin can provide only indirect evidence of availability in the target organ. There exist several methods enabling the localization and distribution of substances within the skin or specific tissue layers (2). Of these, determination of drug distribution in the stratum corneum (SC) is a useful procedure, which can indicate the amount available to penetrate the deeper layers and may provide insight into the likely kinetics of the drug in the skin (3). Until recently, however, such measurements have not always been feasible *in vivo*, often requiring invasive and laborious procedures. Improved methodology has enabled substantial flexibility; for example, correlation of the so-called "stratum corneum reservoir" with *in vivo* percutaneous absorption has been established for a range of chemicals in both hairless rats and humans (4,5). The derivation of quantitative information has, however, relied on the use of radiolabeled drug, a procedure which continues to pose legal and ethical limitations on human *in vivo* experimentation.

Here we report the application of attenuated total reflectance Fourier transform infrared spectroscopy (ATR-FTIR) to obtain a quantitative concentration profile of a model permeant in human SC, *in vivo*. This technique has been previously used *in vivo* to investigate the molecular basis of skin barrier function and penetration enhancer action (6–8). The *in vivo* estimation of water (9) and oleic acid content (8) in the outer region of human SC has also been demonstrated. More recently, a noninvasive assessment of the percutaneous absorption of a model penetrant (4-cyanophenol) as a function of time, was described (7). In addition, the effect of coadministration with the penetration enhancer, oleic acid, was also evaluated. Pursuing these studies, the research presented in this paper was designed to validate this novel *in vivo* method of drug quantification in the SC.

MATERIALS AND METHODS

ATR-FTIR

The basic principles of the ATR-FTIR technique with reference to *in vivo* cutaneous measurements have been previously described (6,9). Spectral measurements were made with an Analect FX-6200 Fourier transform-infrared (FTIR) spectrophotometer (Laser Precision Devices, Irvine, CA) equipped with a liquid nitrogen-cooled mercury–cadmium–telluride detector. The sampling compartment comprised an attenuated total reflectance (ATR) accessory (Spectra-Tech, Stamford, CT), replacing the conventional transmission cell mount. This ATR device supported a trapezoidal internal reflection element (IRE) in a horizontal orientation, enabling the comfortable and reproducible positioning of the subject's forearm onto the optics. For this study, we used a zinc selenide IRE (dimensions, 7.5 × 1 × 0.2 cm) having a refractive index at 1000 cm^{-1} of 2.4 and 60° entrance and exit faces. Under these conditions, and in the spectral region of interest (3000–2000 cm^{-1}), the depth into the SC [refractive index ≈ 1.6 (10)], at which the incident intensity decreases to 37% (or $1/e$) of its value at the interface, is approximately 0.6 μm (11). In practice, this value is accepted as the apparent

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depth of penetration. Spectra obtained represented an average of 40 scans, over a wavelength range of 4000–700 cm^{-1}, acquired during a period of about 70 sec. All measurements were conducted under ambient laboratory conditions (temperature, 21 ± 1°C; relative humidity, 30–40%).

In Vivo Measurements

The *in vivo* studies (approved by the UCSF Committee on Human Research) were conducted in healthy volunteers following their written consent. The subjects (aged 25–45 years) had no history of dermatological disease and were required to maintain the skin sites under investigation (on the midventral forearm) free from application of topical formulations. Prior to the acquisition of a pretreatment (control) IR spectrum, the experimental sites were gently cleansed with water and dried using cotton swabs (Q-tips). The skin was then treated topically with 0.5 mL of either a 10% (w/v) solution of 4-cyanophenol (CP; Aldrich Chemical Co., Milwaukee, WI) in propylene glycol or an identical solution, which also contained 5% (v/v) oleic (*cis*-9-octadecenoic) acid (Sigma, St. Louis, MO). Radiochemical quantification of CP distribution across the SC was achieved by incorporating ^{14}C-labeled CP (Moravek Biochemicals, Brea, CA) into the above solutions, resulting in a final specific activity of 4.4×10^4 dpm/mg. The appropriate formulation was administered via a gauze pad (2 × 8 cm; Webril, Kendall, Boston, MA), which was affixed to the skin with a nonocclusive dressing (Tegaderm, 3M, St. Paul, MN) for a period of 1, 2, or 3 hr. At the end of the treatment period, the pad was removed and the skin surface cleansed of any residual material using ethanol-saturated cotton swabs. An IR spectrum of the dosed site was then recorded. Following this initial spectral acquisition, the site was tape-stripped sequentially using Scotch Book Tape No. 845 (3M, St. Paul, MN), and further IR spectra were obtained at each newly exposed skin surface, up to a maximum of 20 times. In addition, the weight of SC removed by each tape-stripping was determined to define the location of the spectroscopic and radiochemical measurements within the membrane. The tape-strips were subsequently immersed in scintillation fluid for 48 hr and thereafter subjected to liquid scintillation counting (Beckman LS 1000, CA) to evaluate the absolute amount of CP as a function of depth into the SC.

Calibration

Spectroscopic quantification of CP distribution within the membrane is based upon the assumption that IR absorbance is linearly proportional to the concentration of absorbing species. To verify this point, a simple calibration curve was constructed. A series of solutions of increasing concentrations of CP in propylene glycol (1–6%, w/v) was prepared. An aliquot (5 μL) was spread onto the IRE and covered with a glass microscope slide, after which an IR spectrum was recorded. This volume was just sufficient to cover the entire IRE surface without undue leakage over the edges of the crystal. The procedure was performed in quadruplicate for each concentration.

RESULTS AND DISCUSSION

Spectroscopy

The intense C≡N stretching absorbance from CP at 2230 cm^{-1}, in a region where the SC absorbs little IR radiation (12,13), enabled the facile spectroscopic detection of this compound following application to the skin. Since the integrated intensity (II) or area under the curve of the IR absorbance is directly proportional to the concentration of the absorbing species (14,15), the magnitude of this absorbance may be used to quantify the relative amounts of CP in the SC at any given time. However, in ATR-IR, dependence of the signal intensity on the degree of sample/IRE contact, necessitates the use of a normalization procedure. For the purposes of this study, the CP absorbance was integrated between the limits of 2199.8 and 2244.6 cm^{-1}, then ratioed against the corresponding area between the zero and the spectral baselines, as previously described (6), to thus eliminate inter- and intrasubject variabilities in the level of skin-IRE contact established. The relationship between IR absorbance (Abs) and concentration (c) was further substantiated by the *in vitro* calibration with a series of solutions of increasing concentration. This yielded a linear correlation ($Abs = 0.04 + 0.11c$; $r^2 = 0.96$), analogous to a Beer–Lambert relationship.

To generate drug distribution profiles as a function of SC depth, successive layers were progressively revealed by multiple tape-stripping, prior to spectroscopic analysis. Although the depth of SC penetration achieved by successive tape-strippings cannot be measured directly, the distance into the membrane can be related to the weight of SC removed. The basis for this assertion is that one can write

$$\text{mass} = \text{volume} \times \text{density} = (\text{depth} \times \text{area}) \times \text{density}$$
$$\Rightarrow \text{mass} \propto \text{depth}$$

with the assumptions that (a) SC density does not vary with depth and (b) experimentally, a constant area of SC is tape-stripped. Consequently, drug distribution profiles presented here have been expressed as a function of the cumulative SC weight removed.

Plots illustrating the weight of SC removed tape-strip following exposure to CP formulations (with and without oleic acid) for 1, 2, and 3 hr, are depicted in Fig. 1. Treatment of the skin for 1 hr, with the CP formulation containing OA, did not significantly alter the weight of SC removed compared to treatment with the control. However the amounts removed following 2- and 3-hr applications of the OA-containing formulations were significantly greater than the corresponding controls. In fact, treating the skin for 3 hr with the fatty acid formulation severely undermined SC integrity. If the SC is visualized as corneocyte "bricks" embedded in a lipoidal "mortar," the disruptive effect of OA on the SC may be likened to a "brick loosening" effect, presumably mediated via OA disruption of the intercellular domain. As previously noted, the initial tape-strips removed somewhat more material than subsequent ones (6). This may be a result of the reduced cohesiveness of the outermost, desquamating layers of the SC. Increasing the application time of the formulations progressively increased the weight of skin removed, an observation which may be attributed to the correspondingly elevated state of skin hydration (16). The results clearly illustrate that the amount of SC adhering to each consecutive tape-strip is variable, depending on the experimental conditions. Consequently, this should be taken into consideration when interpreting SC drug distribution pro-

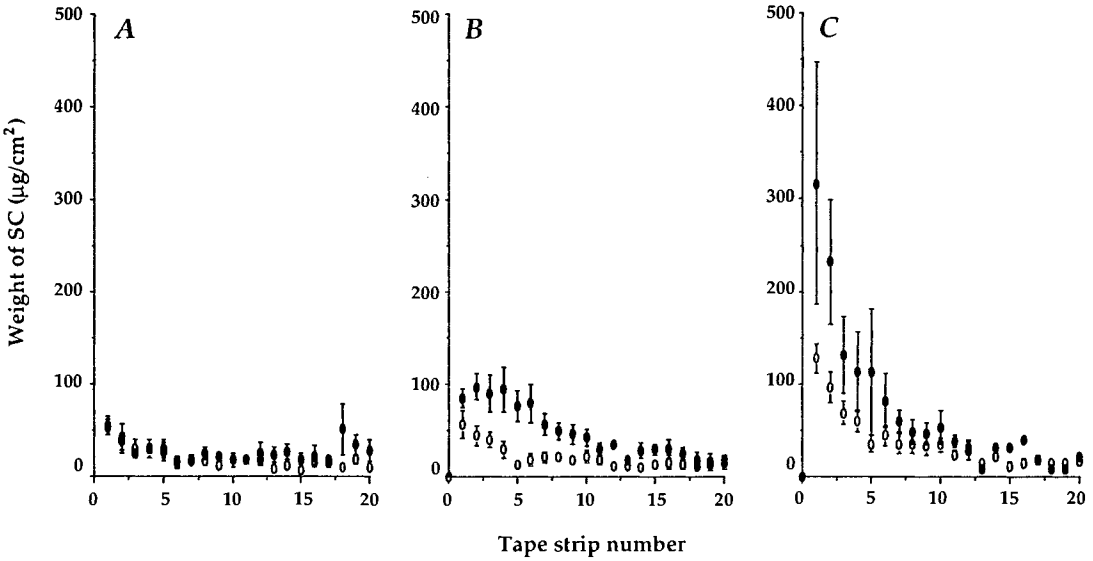


Fig. 1. Weight per unit area of SC removed by sequential tape-stripping, following treatment for (A) 1 hr, (B) 2 hr, and (C) 3 hr with either a 10% (w/v) solution of CP in propylene glycol (○) or the identical solution containing 5% (v/v) oleic acid (●). Mean ± SE; $n = 4$.

files, typically plotted as a function of "tape-strip number," where the linear scale of the x axis implies that each tape-stripping event removes a constant mass of SC and, therefore, corresponds to a uniform increment in SC depth (17,18). The influence of this disproportionality on the perceived concentration profile becomes apparent if the same data set of drug distribution across the SC is plotted as a function of (a) tape strip number and (b) cumulative SC weight (which is proportional to "depth"), as in Fig. 2.

Plots showing the normalized II of the CP-associated IR absorbance (at 2230 cm^{-1}) against the cumulative weight of SC removed, following application of the control, and the OA-containing formulations for periods of 1, 2, and 3 hr are shown in Fig. 3. The influence on the percutaneous penetration of CP, of the two experimental variables, (a) incorporation of OA in the vehicle and (b) varying application time, may be observed from these graphs. In the absence of the enhancer, the delivery of CP into the membrane progressively increases with increasing application time. Incorporation of OA into the formulation further amplifies this effect. Although OA does not modify the distribution of CP across the barrier following a 1-hr treatment, longer exposure of the SC to the enhancer results in (a) significant elevation of the amount of CP in the membrane and (b) delivery of the per-

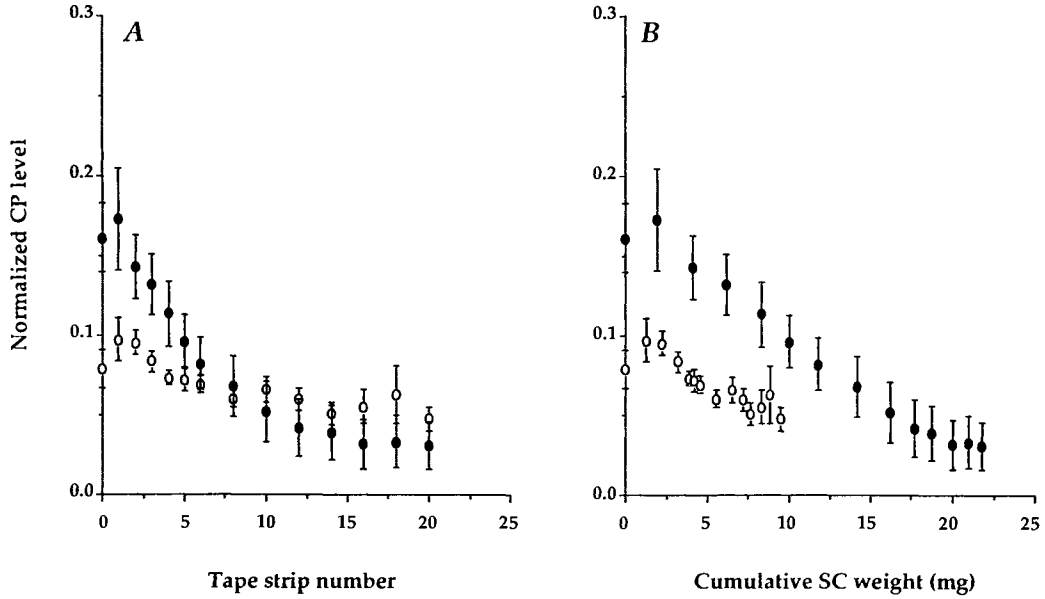


Fig. 2. Plots of the normalized CP level as a function of (A) tape-strip number and (B) cumulative weight of SC removed, following treatment with either a 10% (w/v) solution of CP in propylene glycol (○) or the identical solution containing 5% (v/v) oleic acid (●). Mean ± SE; $n = 4$ or 5.

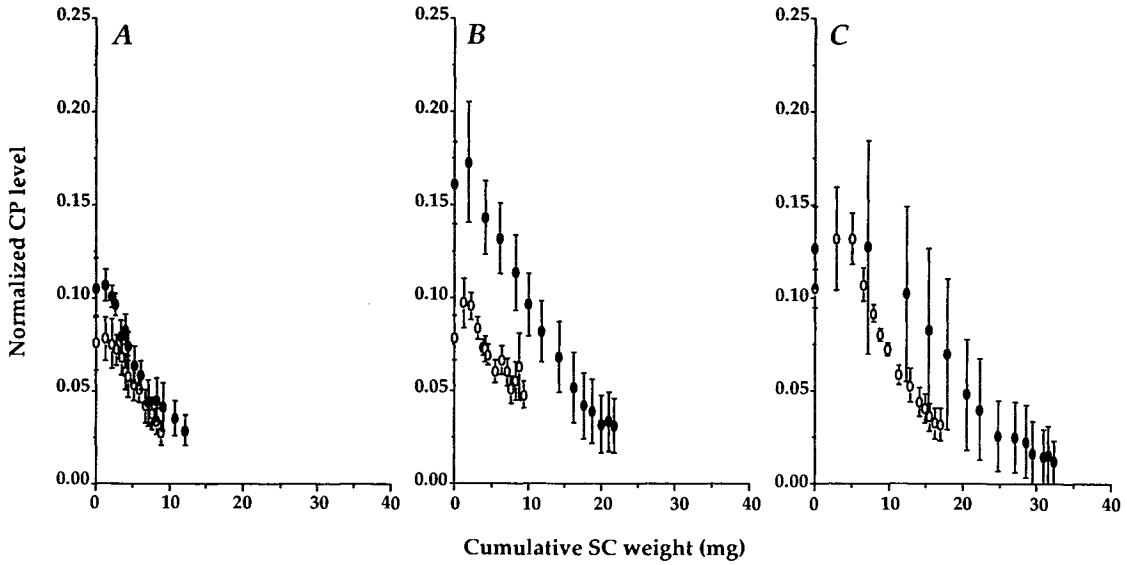


Fig. 3. Normalized CP level as a function of SC weight following treatment with either a 10% (w/v) solution of CP in propylene glycol (○) or the identical solution containing 5% (v/v) oleic acid (●) for (A) 1 hr, (B) 2 hr, and (C) 3 hr. Mean ± SE; n = 4 or 5.

meant "deeper" into the SC, relative to the controls. Implicit in these observations is the ability of OA to facilitate the penetration of CP across the SC and concomitantly enhance the rate of drug delivery. This is consistent with previous reports from our laboratory, demonstrating the hastened clearance of CP from the surface of the SC, in the presence of OA (under identical conditions) (8). These data also revealed that OA did not significantly affect the *in vitro* uptake of CP into the SC, implying a true enhancer effect, as opposed to an improved partitioning phenomenon. The mechanism, by which OA compromises the barrier properties of the SC, remains the subject of considerable interest, with several theories having been proposed (8,19–21). Recently, the manner in which OA may alter the diffusional resistance of the SC was modeled, using *in vivo* data from the literature (22). These simulated profiles suggest that OA alters the diffusivity of the outermost layers preferentially to that of the deeper regions, a scenario which appears to be consistent with the data presented here (Fig. 3).

In essence, these spectroscopic results illustrate a simple and efficient method to monitor, qualitatively, the *in vivo* percutaneous penetration and enhancement of a model permeant. The additional experiments performed in this study were designed to *quantitate* the absorption and enhancement process.

Radiochemical Measurements

Radiolabeled CP (^{14}C-CP) was incorporated into the formulations applied to the skin. By subjecting the SC tapestrips to liquid scintillation counting, absolute amounts of

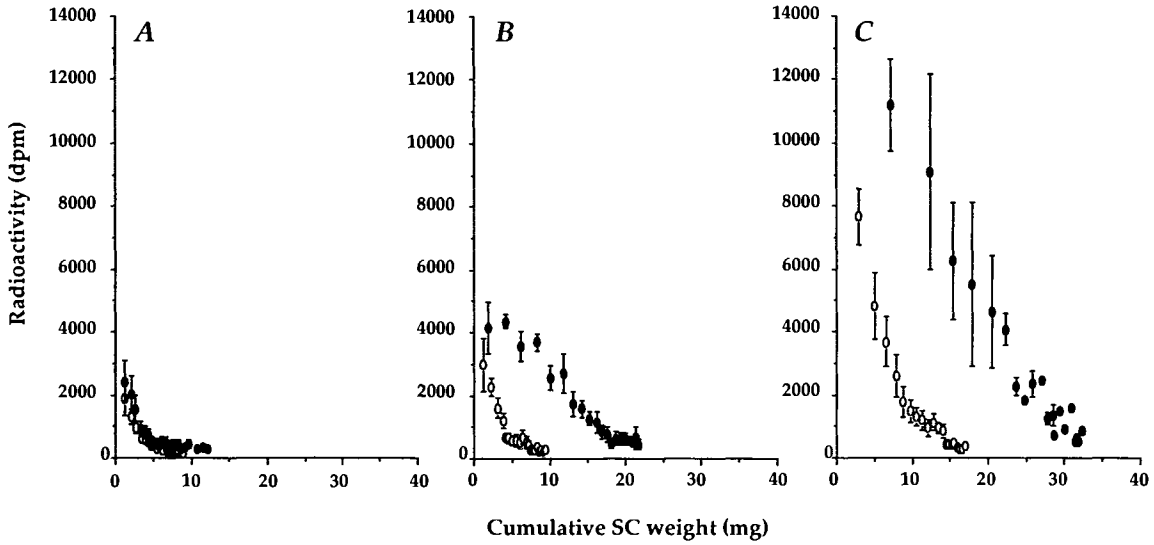
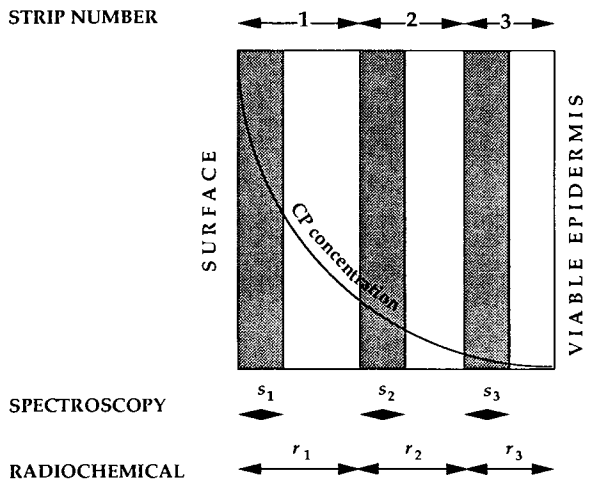


Fig. 4. Distribution of ^{14}C-CP as a function of SC weight, following application of the control (○) and OA treatment (●) formulations for (A) 1 hr, (B) 2 hr, and (C) 3 hr. Mean ± SE; n = 4 or 5.



$$\frac{\text{II}\,(s_1) + \text{II}\,(s_2)}{2} \times \text{Weight of SC (mg)} \propto \text{Radioactivity}\,(r_1)$$

Fig. 5. Schematic representation of the model used to correlate the radiochemically and spectroscopically acquired SC distribution of CP. s_n represents the region of SC monitored by IR. r_n represents the segment of SC removed by tape-stripping and subjected to radiochemical analysis, where n = tape-strip number. The information from the two approaches may be equated according to the given relationship.

drug in the different layers of the SC could be assayed. In this way, radiochemical as well as spectroscopic distribution profiles of CP across the SC, as a function of depth and treatment time, were generated. The results are summarized in Fig. 4 and reveal behavior closely parallel to the spectroscopic data in Fig. 3. The effects of increasing application time and OA exposure are remarkably reproduced in the radiochemically derived data.

A schematic representation of data acquisition as a function of SC depth, using the two techniques, is shown in Fig. 5. The spectroscopic data are collected from the skin surface region exposed to the IRE, the depth of penetration being determined by the relative refractive indices of the IRE and sample and by the incident angle of radiation. In this study, each IR spectrum samples on the order of 1 μm into the membrane. In contrast, the radiochemical assay determines the absolute amount of drug present in that fraction of the SC which separates each sequential pair of IR observations. One cannot assume (nor prove) that any particular tape-strip removes *exactly* that portion of SC "seen" by the immediately preceding IR measurement. Therefore, in correlating the two measurements, it is necessary to plot the average of each pair of spectroscopic measurements, corrected for the SC weight removed, against the radioactivity (r) in the intervening tape-strip (Fig. 5).

Figure 6 presents plots of the radioactivity data obtained from tape-stripping versus the corresponding spectroscopic measurements. Excellent correlation between the two approaches (r^2 values of 0.84, 0.93, and 0.90 for 1-, 2-, and 3-hr treatments, respectively) are obtained, indicating the potential of the IR technique to quantify, accurately, *in vivo*, the distribution of a topically administered drug in the SC. The key advantage of spectroscopic quantification is evident: elimination of the need for a radiolabeled marker, thus circumventing the legal and ethical limitations posed by their use in humans. Clearly, an external calibration correlating the observed IR signal to concentration is necessary to accomplish this goal. We have described the linear relationship between drug concentration and normalized IR absorbance above; similarly, one can envisage performing an appropriate calibration of this type to quantify drug penetrant levels in

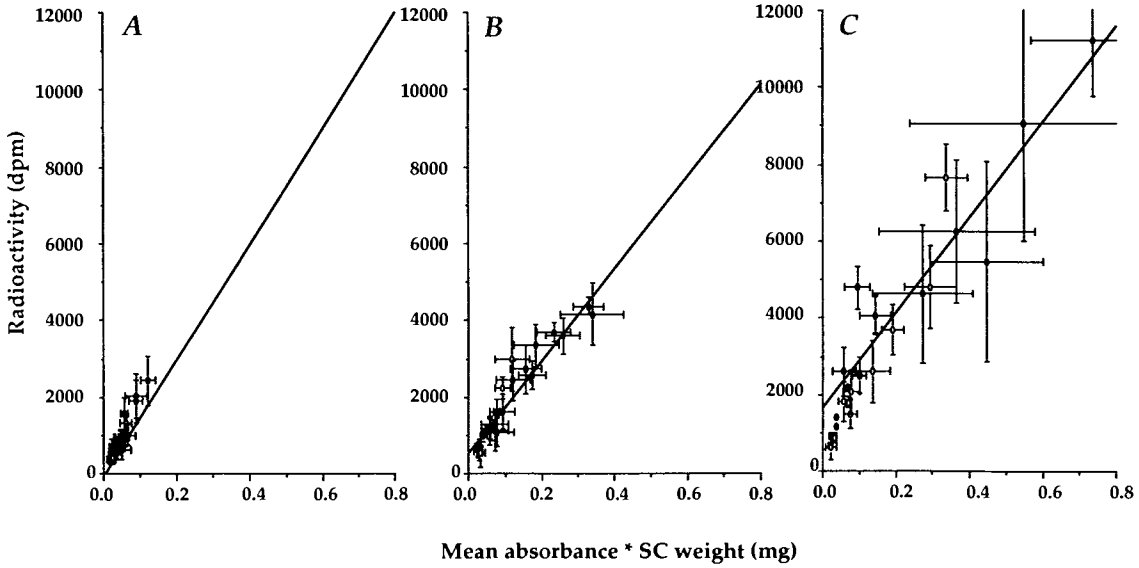


Fig. 6. Correlation between the absolute radiochemical measurement of CP distribution across the SC and the corresponding spectroscopically determined level, following (A) 1-hr, (B) 2-hr, and (C) 3-hr application of the control (○) and OA treatment (●) formulations. Mean ± SE; n = 4 or 5. The IR absorbance has been normalized to account for the different depths of SC sampled by the radiochemical and spectroscopic techniques. Refer to Fig. 5 and accompanying text.

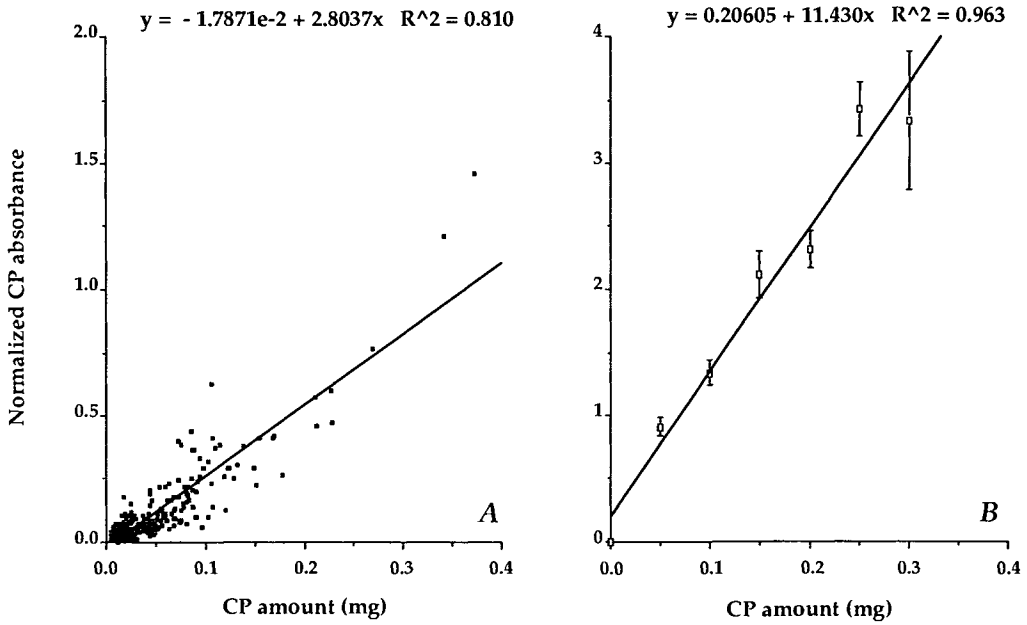


Fig. 7. Correlation between the normalized CP absorbance and CP level, as obtained from (A) the *in vivo* radiochemical distribution profile and (B) an "*in vitro*" calibration. The data represent the mean ± SE (n = 4) in the case of profile B.

the SC *in vivo*. To examine the validity of this approach, the relationship between IR absorbance and drug level obtained from the above-mentioned "*in vitro*" calibration was compared to the "*in vivo*" correlative data of IR absorbance versus radioactivity (and, hence, amount of drug) represented in Fig. 6. Individual radioactivity data points from Fig. 6 were transformed (disintegrations per minute → amount) and pooled to provide a plot of normalized IR absorbance versus amount of CP (Fig. 7A). The *in vitro* calibration data were similarly transformed to yield a plot of normalized IR absorbance (corrected for weight of solvent present on crystal) versus the total amount of CP applied to the crystal (Fig. 7B). The ability of the *in vitro* calibration to predict drug concentration from the spectroscopic absorbance values may be assessed from the relative magnitudes of the slopes of these two graphs (identical slopes indicating an "ideal" calibration). Experimentally, such ideality is unlikely to be observed since the precise proportionality between absorbance and concentration will be dependent upon the nature of the medium employed. The dissimilar media in our experiments (SC in A and PG in B) will influence this relationship by virtue of their differing refractive indices and light scattering properties. The results presented here suggest that a calibration of this type is able to estimate the drug level in the SC to within a factor of 5 of the true value. The origins of this difference cannot be uniquely identified at this time. In addition to the factors discussed above, it should also be noted that the "calibration plot" in Fig. 7B would benefit from more data in the 0- to 0.2-mg range, which is of the most relevance to the likely *in vivo* application of this technique. Furthermore, it is important to establish the extent to which the proposed approach is a function of the vehicle employed; i.e., To what degree are the optical properties (*viz.*, refractive index) of the SC modified by the presence of different formulation components (and, in consequence, is the approach going to be highly vehicle-specific)? Obviously, validation experiments of this nature need to be performed with a variety of drugs and conditions before strong endorsement of this approach can be claimed. Nevertheless, the current results are promising and reveal the capacity of the IR technique to furnish quantitative, as well as qualitative information.

In summary, the exceptional similarity and correlation between data obtained from two independent and complementary methods (radiochemical and spectroscopic) provide initial validation for the technique of reflectance IR spectroscopy as a means of quantitative analysis. It would appear that with appropriate calibration, such data can be obtained without the use of a radiolabeled marker. Considerable basic knowledge should evolve, therefore, from experiments of this type; significantly, the data pertain to human skin *in vivo*. From a practical standpoint, the approach may find application in the measurement of topical drug bioavailability and formulation bioequivalence.

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