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METHODS OF ASSESSING THE PERCUTANEOUS ABSORPTION OF VOLATILE CHEMICALS IN ISOLATED PERFUSED SKIN: STUDIES WITH CHLOROPENTAFLUOROBENZENE AND DICHLOROBENZENE

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The experimental determination of dermal absorption of volatile chemicals is fraught with difficulties. The isolated perfused porcine skin flap (IPPSF) is a biologically intact, perfused skin preparation that has been employed to predict dermal absorption of chemicals in humans. The purpose of this work was to explore various experimental dosing strategies for volatile chemicals using dichlorobenzene (DCB) and chloropentafluorobenzene (CPFB) as model compounds. Effects of complete occlusion and various strategies of vapor trapping, vapor dosing, and solvent effects were explored. The results suggest that dosing methodology is a major determinant of dermal absorption and could easily skew results obtained from different systems. A biologically sensitive system such as the IPPSF is particularly sensitive to the manipulations required to ensure precise dosing of these compounds. An interesting finding was that the effects of solvents on compound absorption that are routinely described in liquid dosing scenarios were also detected when both the compound and solvent were exposed during the vapor phase.

Keywords chloropentafluorobenzene, dermal absorption, dichlorobenzene, volatile chemicals

The assessment of the percutaneous absorption and penetration of volatile compounds is experimentally challenging. In many cases, chemical loss is avoided by conducting studies under conditions of occlusion to protect against loss of dose. However, this scenario often does not model

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the natural exposure conditions, and this poses problems when making risk assessment calculations. Complete occlusion of a dosing site alters absorption profiles (Chang and Riviere, 1993). The isolated perfused porcine skin flap (IPPSF) is an in vitro model system that has been utilized to model compound absorption and skin disposition (Riviere et al., 1986, 1995; Riviere and Monteiro-Riviere, 1991). This model has been validated as being predictive of in vivo human absorption for a large and diverse series of compounds (Riviere et al., 1992, 1995; Wester et al., 1998). The advantage of the system is that all the biological components of skin that could modulate percutaneous absorption (e.g., the stratum corneum barrier, epidermal metabolism, dermal vascular uptake, irritation) are functional in this perfused tissue preparation.

Risk assessment of volatile compounds is generally focused on their primary route of exposure, inhalation. As a result, there are few data available in the literature concerning dermal absorption after topical exposure, as might occur when the skin surface is exposed to a compound. Dichlorobenzene (DCB) is commercially utilized as a space deodorizer, moth repellent, and fungicide. The compound is also used as an intermediate in the synthesis of resins, organic solvents, pesticides, and dyes (ATSDR, 1993). A recent epidemiological survey suggests that 96% of the adult population in the United States has detectable levels of DCB in their blood (Hill et al., 1995). At very high doses, DCB has been associated with kidney and hepatic toxicity and is a putative carcinogen in laboratory animal studies. Its biotransformation has been investigated in the context of its role as a carcinogen (Hissink et al., 1997a, b). Chloropentafluorobenzene (CPFB) is a volatile fluorinated hydrocarbon that has been proposed to be used as a chemical warfare agent simulant because it has a very low level of toxicity, being associated only with mild skin and eye irritation in rabbits (Clewell and Jarnot, 1994). For both of these compounds, dermal penetration has not been adequately assessed in a model system predictive of human absorption.

A second variable often encountered in assessing the absorption and cutaneous toxicity of such compounds is the protocol used to dose low concentrations in laboratory animal studies. In most cases, especially when assessing low-level exposure, vehicles must be used to apply test compounds to skin. It is recognized that vehicles may alter the dermal absorption properties of chemicals so that they differ from those that occur in cases of neat application (Löf and Johanson, 1998), a phenomenon documented in IPPSF studies with phenol and paranitrophenol (Brooks and Riviere, 1996).

The purpose of this project was to develop methodologies that could be used to study volatile compound absorption in the IPPSF. Because of the peculiarities of this model (e.g., sensitivity to handling during an experiment, venous drainage open to the environment, tubular shape,

variability in size due to surgical procedure), a new dosing system was developed for the IPPSF that allowed more controlled exposure to these chemicals. The process of conducting these experiments involved (1) development of a cradle chamber to trap the evaporated compound in the area next to the skin, (2) assessment of the amount of chemical absorbed into the perfusate from the chemical that evaporated from excised skin, (3) exposure of the IPPSF to neat test compound and to test compound in a vehicle, (4) assessment of the amount of test compound in the perfusate as a result of exposure to the volatile compound vapor, and (5) development of a dosing dome that allowed dosing a vapor to skin without vapor uptake directly into the perfusate. Model compounds for these experiments were CPFB and DCB, dosed neat and in vehicles.

MATERIALS AND METHODS

Experiment Scenarios

A number of experimental scenarios were set up to investigate the effects of different dosing strategies on compound absorption in the IPPSF. Table 1 lists the experiment scenarios studied. Compounds were applied neat or in varying concentrations in vehicles, generally using $n = 4$ replicates per treatment condition. Ethanol was the vehicle for DCB experiments; however, it was necessary to use acetone in the CPFB experiments because of analytical interference resulting from the fact that the elution times of ethanol and CPFB are similar during gas chromatography (GC) analysis. In scenario A, compound was dosed on IPPSFs perfused in open chambers. In scenario B, compound was dosed on the IPPSF and the entire dosing site was immediately occluded with

TABLE 1. Experimental Scenarios Investigated

Protocol number	Compound	Exposure conditions	Dose	Vehicle	*n*
A	CPFB	Nonoccluded	20 μL liquid	Neat	4
B	CPFB	Occluded patch	20 μL liquid	Neat	4
C	CPFB	Cradle chamber	20 μL liquid	Neat	4
D	CPFB	Cradle chamber (excised skin)	20 μL liquid	Neat	4
E	CPFB	Cradle chamber	20 μL liquid	Neat	6
F	CPFB	Cradle chamber	2 μL liquid	Neat	4
G	CPFB	Cradle chamber	2 μL liquid	8 μL acetone	4
H	CPFB	Cradle chamber	10 μL liquid	10 μL acetone	4
I	DCB	Cradle chamber	20 μL liquid	Neat	4
J	CPFB	Dosing dome	20 μL vapor	Neat	7
K	DCB	Dosing dome	20 μL vapor	Neat	4
L	DCB	Dosing dome	50 μL vapor	Neat	4
M	DCB	Dosing dome	10 μL vapor	10 μL ethanol	3
N	DCB	Dosing dome	20 μL vapor	20 μL ethanol	2

cellophane tape. In C, compound was dosed using the cradle chamber as described later. In D, compound was dosed onto excised, nonperfused skin in a cradle chamber, with perfusate flowing through the chamber in order to assess evaporation into perfusate. Treatments E, F, G, and H compared CPFB absorption in the cradle chamber at two doses and coadministered in acetone vehicles. Treatment I assessed DCB absorption in the cradle chamber. Treatments J, K, L, M, and N utilized the dosing dome described later and assessed neat CPFB and DCB at two doses in ethanol vehicles.

Isolated Perfused Porcine Skin Flap

The routine two-stage surgical procedure was used to create IPPSFs (Riviere et al., 1986). The flap was then transferred to a temperature- and humidity-regulated perfusion system. In the chamber, the perfusion flow rate, pH, temperature, and relative humidity were maintained at a perfusation flow rate of 1 mL/min/flap (3–7 mL/min/100 g tissue), a pH of 7.4, a temperature of 37°C, and a relative humidity of 60 to 80% in a nonrecirculating perfusion configuration. Flow rate and perfusion pressure (arterial, 30–70 mmHg) were recorded every 30 min during experiments. The perfusion medium was a Krebs-Ringer bicarbonate buffer (pH 7.4, 350 mOsm/kg) containing albumin (45 g/L) and glucose (80–120 mg/dL).

Figure 1A depicts the configuration of the cradle chamber, which allowed the volatile compound to evaporate and be trapped in an environment around the skin flap. A 1 × 3-cm dosing area was drawn on the surface of the skin flap. The maximum dose that could be applied to this area was 20 μL. The cradle chamber was then secured in place with a parafilm gasket. The cannula end of the cradle was sealed to minimize evaporation from the cradle chamber. The skin flap was dosed via the slit in the top of the cradle chamber, and the slit was immediately covered with tape. Vapor samples (0.5 mL) were taken from this slit via a 25-gauge needle through the tape, and the hole in the tape was sealed with tape immediately after sampling. Care was taken at every step to minimize loss of the vapor of the test compound. Perfusate entered the arterial supply via the cannula and the venous drainage was collected in a funnel built into the floor of the cradle because cannulation of the draining veins is not feasible in routine studies. This configuration could result in exposure of perfusate to dosed vapor, so nonperfused skin wrapped on a tubular stint was exposed to compound and air, and perfusate samples were monitored over time. These data suggested that this configuration would require a normalization procedure for accurate assessment of skin penetration.

Figure 1B depicts the second approach, which utilized a glass dosing dome that restricted vapor contact to dosed skin. This eliminated the

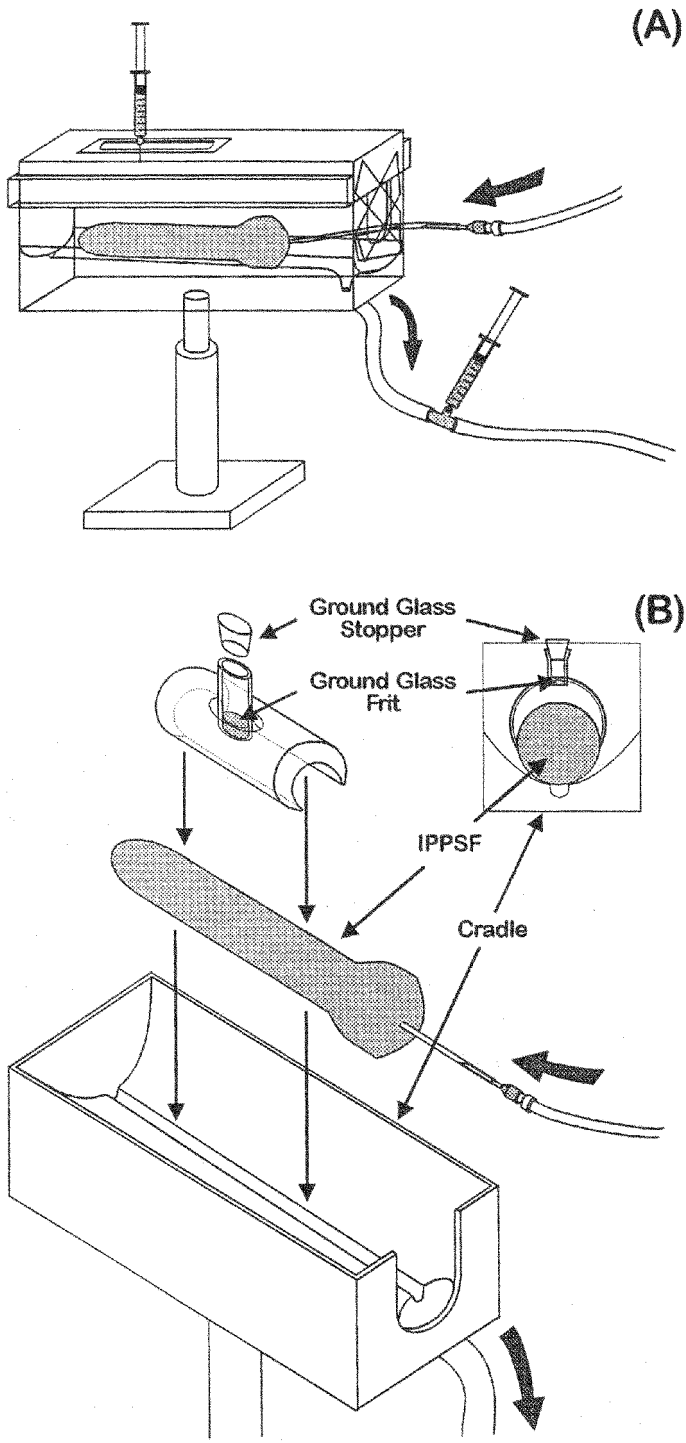

FIGURE 1. Dosing chambers assessed on IPPSFs. A. Cradle chamber dosing allows a volatile chemical to evaporate from the dosing site and be trapped in the environment around the skin flap. B. The glass dosing dome exposes a defined skin surface area to volatilized compound that is applied at the ground glass frit.

need to normalize perfusate concentrations for vapor contact. Several sizes of glass dosing domes have been developed to accommodate various skin flap sizes. (Although care is taken to produce skin flaps that are the same length and diameter, differences in donor pigs and surgical procedures make it impossible to produce absolutely uniform-sized skin flaps.) The glass dosing dome was placed over the skin flap, and the snugness of the fit held the dome in place without overly constricting the flap, which would produce perfusion problems. The ground-glass stopper was removed from the glass dosing dome, and the dose of the volatile test compound was applied to the porous ground-glass frit at the base of the central tube. The glass stopper was replaced immediately after the dose was pipetted. The test compound then volatilized to fill the dome with test compound vapor. Because the glass dosing dome eliminated direct contact between the vapor and the perfusate, the test compound that appeared in the perfusate resulted solely from absorption through the skin.

At termination, several samples were taken for mass balance of the test compounds. The surface of the dosed area was swabbed twice with gauze moistened with a 1% soap solution followed by 12 stratum corneum tape strips. The entire dosed area was removed. A 1 cm × 1 cm core of the dose area was removed and frozen for subsequent studies of depth of penetration. The remaining dosed area was extracted using the appropriate solvent and analyzed for test compound. A sample of the fat under the dosed area and a 1 cm × 1 cm-area of nondosed skin were extracted and analyzed.

Gas Chromatography

Sample analysis was made by means of gas chromatography using a Hewlett Packard 5890 II gas chromatograph and an external standard method. DCB samples were extracted from perfusate and tissues using iso-octane, and CPFB samples using hexane. Air samples were directly injected. An Alltech Capillary Column (SE-30 15 M × 0.53 mm) (Alltech Assoc., Inc., Deerfield, IL) with a column flow rate of 10 mL/min was used; it produced a total flow (column + auxillary) of 50 mL/min. The carrier was 95% argon and 5% methane. The oven temperature was 70°C, the injector temperature 180°C, and the electron capture detector temperature 380°C. Peaks were quantitated using the external standard area method and linear calibration curves for air, perfusate, and tissue samples.

All data are expressed as mean ± SEM.

RESULTS AND DISCUSSION

Figure 2A illustrates the mean CPFB perfusate values over time when IPPSFs were dosed and not occluded (scenario A), placed under an occlusive dressing (scenario B), and placed in the cradle chamber (scenario C) (Table 1). Figure 2B depicts the mean CPFB va

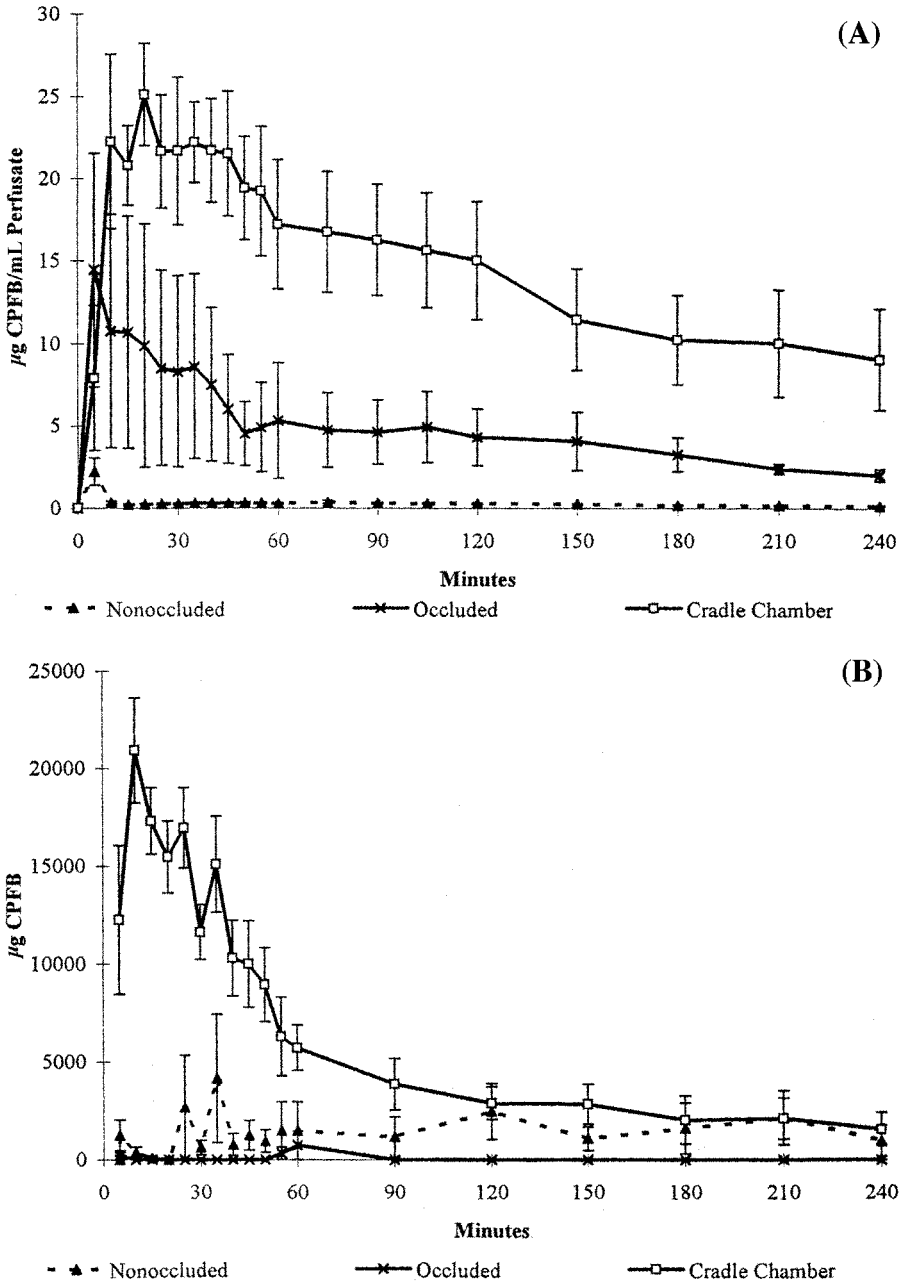

FIGURE 2. CPFB absorption into IPPSF perfusate after dosing in nonoccluded and occluded environments and in the cradle chamber. A. The absorbed dose is expressed as a concentration in perfusate. B. Total mass absorbed from the vapor. Mean ± SEM.

simultaneously collected for the same experiments. Vapor samples were taken from the area over the IPPSF in the nonocluded and occluded systems and from the sampling port in the cradle cha

A number of observations can be made based on this first series of exposures. The perfusate data demonstrate that only trace amounts of CPFB were absorbed from a totally nonoccluded system (62.4 ± 11.4 μg). CPFB could be detected in the air sampled from the IPPSF chamber despite its relatively large volume (approximately 1 cu. yd). In contrast, occluded dosing produced a rapid increase in perfusate levels and a larger total absorption (1070 ± 477 μg). The lack of continuously detectable CPFB in the vapor samples under occluded dosing supports the efficiency of the occlusion process. The surprising data, which prompted many of the studies in this manuscript, were the high levels of absorption seen when CPFB was dosed in the cradle chamber (2892 ± 620 μg). However, this was accompanied by high vapor concentrations in the cradle chamber. Theoretically, dosing with the use of a completely occlusive dressing should produce maximal absorption. The higher absorption seen in the cradle chamber prompted the hypothesis that absorption was occurring by two routes, through skin at the dosing site and into perfusate from the chamber environment as it exited the flap and collected in the drainage funnel. This exposure of perfusate to exposed vapor was a major limitation of the system and prompted remaining studies to investigate how such data could be handled.

To assess this possibility, in scenario D, CPFB was dosed onto an excised piece of dermatomed pig skin (500 μm thick) applied over a glass cylinder to reproduce the geometry of the IPPSF. This skin was not perfused, so any CPFB appearing in the perfusate must have been secondary to absorption from the cradle chamber environment. In this exposure scenario, total absorption into the perfusate was 1114 ± 349 μg, a value approximately 40% that seen when a perfused IPPSF was dosed. The difference (1778 μg) approached the value seen under occluded dosing.

These observations prompted a second series of studies, scenarios E though H, in which CPFB was dosed using cradle chambers while perfusate and cradle chamber while concentrations were simultaneously monitored. In these studies, additional care was taken to avoid compound loss due to leakage and evaporation from samples. Figure 3A illustrates the mean vapor levels of CPFB inside the cradle chamber over time for four different dosing protocols. Figure 3B illustrates the mean CPFB values in the perfusate. Figure 3C depicts the ratio of CPFB in perfusate divided by CPFB in vapor. A clear plateau in this ratio occurred within 10 min. The following computational approach was then utilized to generate the corrected perfusate absorption profiles depicted in Figure 3D:

1. Determine μg/mL concentrations in vapor and perfusate samples.
2. Determine ratio [Perfusate(μg/mL)]/[Vapor(μg/mL)].

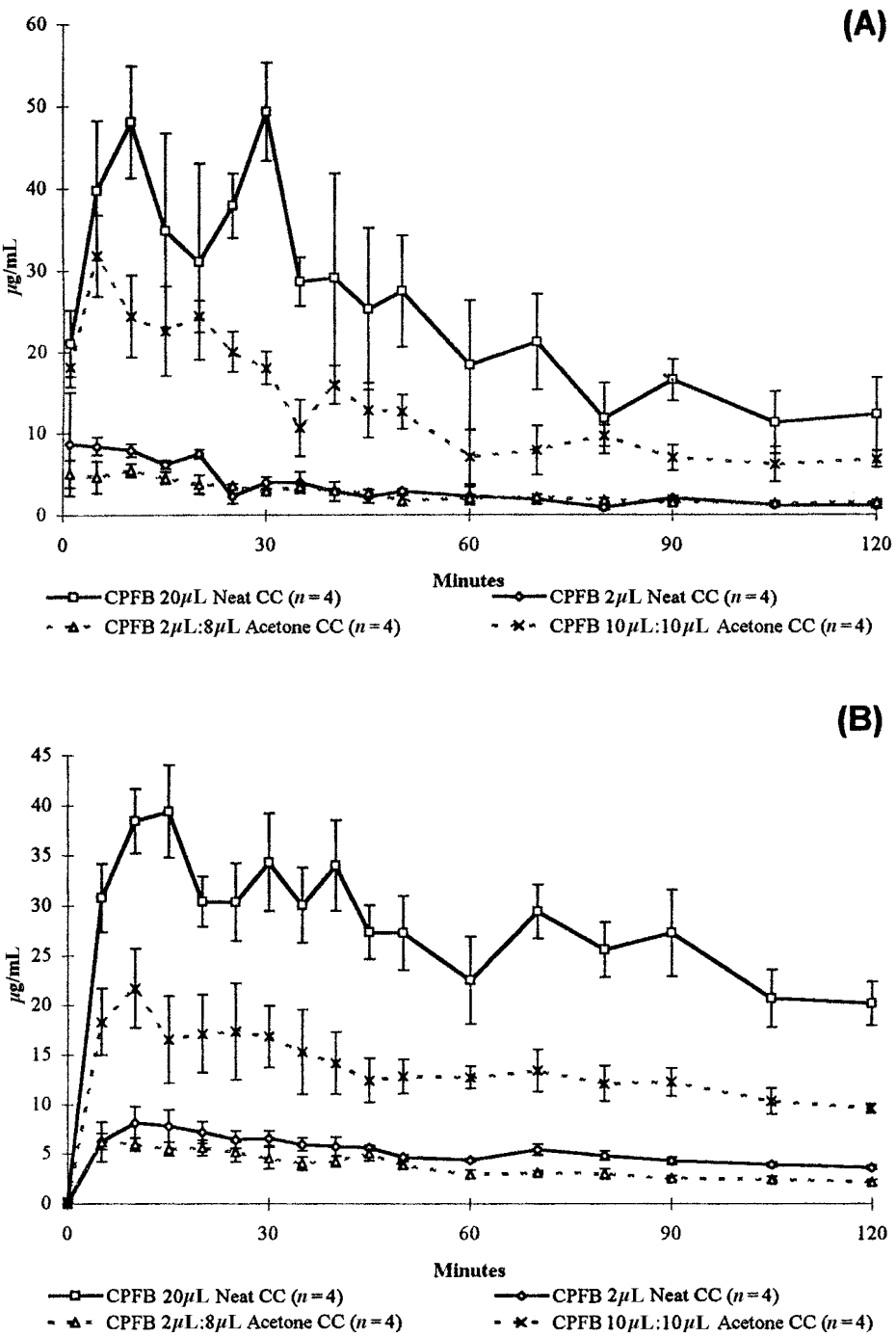

FIGURE 3. Percutaneous absorption of CPFB dosed according to four treatments: 20 μL neat; 2 μL neat; 10 μL CPFB and 10 μL acetone; 2 μL CPFB and 8 μL acetone. A. Concentration in cradle chamber atmosphere. B. Concentration in IPPSF perfusate. *(Continued)*

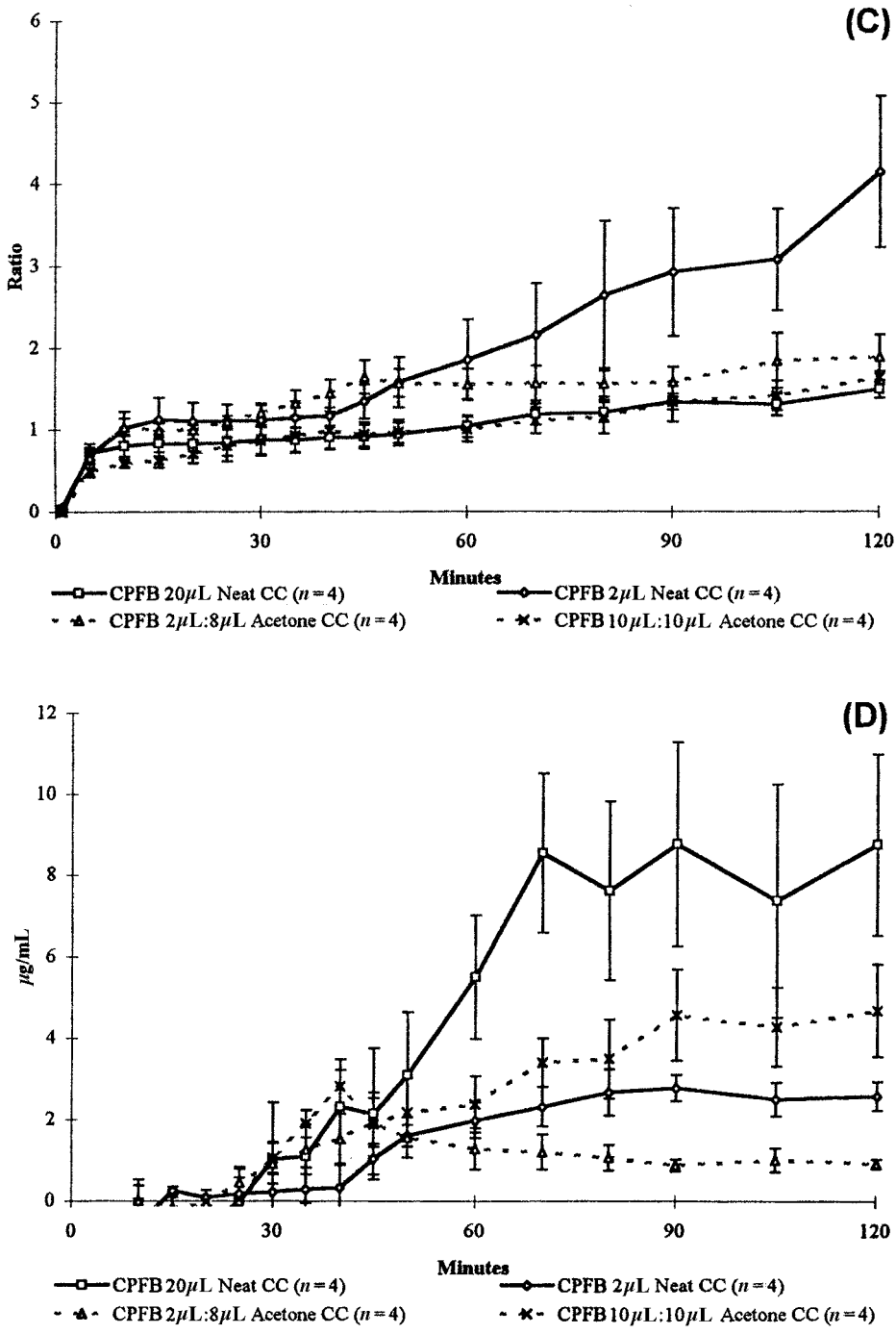

FIGURE 3. (Continued) C. Ratio of CPFB in perfusate to vapor. D. Corrected perfusate concentrations using equation. CC, cradle chamber.

TABLE 2. Percutaneous Absorption of CPFB Liquid Dosed Using Cradle Chambers

	Actual dose	Total absorption into perfusate		Absorption into perfusate from air		Corrected dermal absorption	
Treatment	(μg)	(μg)	(%D)	(μg/mL)	(%D/mL)	(μg)	(%D)
20 μL neat	31,360	3634^{a} (514)	$12^{b,c}$ (2)	700^{a} (121)	2.2^{a} (0.14)	2934^{a}	0.81^{a}
10 μL CPFB/ 10 μL acetone	15,680	1857^{b} (287)	$12^{b,c}$ (2)	$409^{b,c}$ (46)	2.6^{a} (0.3)	1448^{b}	0.78^{a}
2 μL neat	3136	664^{c} (49)	21^{a} 2	86^{d} (7)	2.7^{a} (0.2)	578^{c}	0.18^{b}
2 μL CPFB/ 8 μL acetone	3136	482^{c} (69)	15^{b} (2)	81^{d} (8)	2.6^{a} (0.2)	401^{c}	0.13^{b}

Note. Values are mean (SEM). D, dose.

^{a,b,c,d}Data with superscripts are compared within columns. Values with the same letter are not significantly different.

3. Determine first plateau of ratios = ratio*.
4. Determine corrected [perfusate] using the following equations:

$$\text{Ratio}^{*} = [\text{perfusate}] / [\text{vapor}]$$

$$[\text{Perfusate}] = (\text{ratio}^{*} \times [\text{vapor}])$$

$$\text{Corrected } [\text{perfusate}_{0-120}] = [\text{original perfusate}_{0-120}] - (\text{ratio}^{*} \times [\text{vapor}_{0-120}])$$

The corrected absorption parameters are listed in Table 2. An insignificant fraction of the applied dose was recovered in any of the stratum corneum tape strips, dosed skin, or fat samples collected. The absorption of CPFB from the cradle chamber into the perfusate was relatively constant across all scenarios (approximately 2.5%). The rank order of absorption was 20 μL neat CPFB >10 μL CPFB in 10 μL acetone >2 μL neat CPFB >2 μL CPFB in 8 μL acetone. As can be seen by comparing absorption with percentage of applied dose, absorption efficiency decreased with higher doses. Dosing in an acetone vehicle reduced the absorption of the lower dose.

In contrast to the highly volatile CPFB, DCB absorption profiles (Figures 4A through D) using this exposure scenario (I) had a minimal vapor effect, and any correction did not significantly alter the absorption profile. Total perfusate absorption was 616 $\pm$ 31 μg. Concentrations were detectable in tissue samples but were uniformly less than 5% of perfusate absorption at the end of an experiment. It is to be noted that in contrast to CPFB, absorption did not plateau with DCB.

There was a great deal of variability in these studies, partially attributable to the difficulty of achieving a constant dosing surface area.

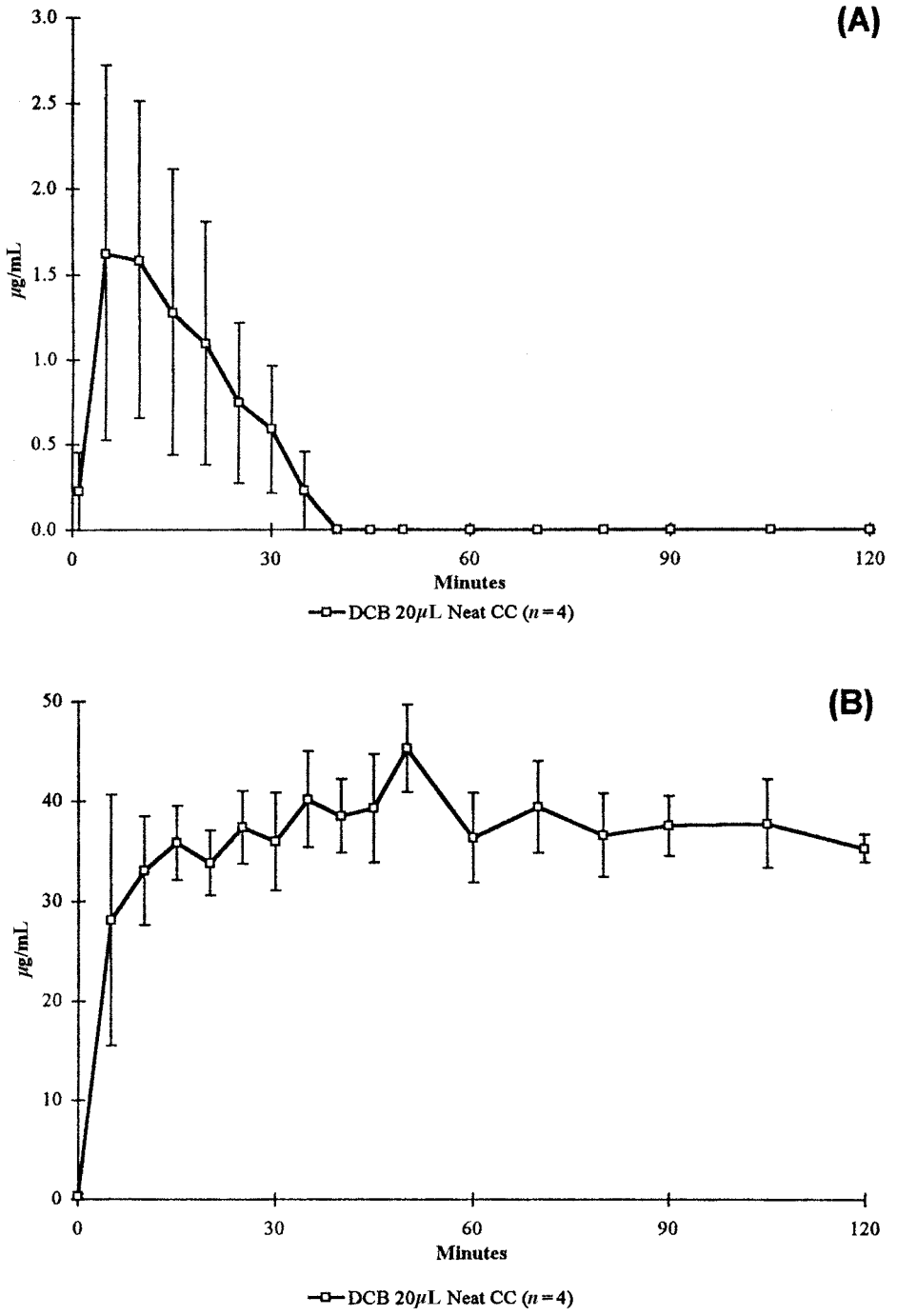

FIGURE 4. Percutaneous absorption of DCB by IPPSF. A. Concentration in cradle chamber atmosphere . B. Concentration in perfusate. *(Continued)*

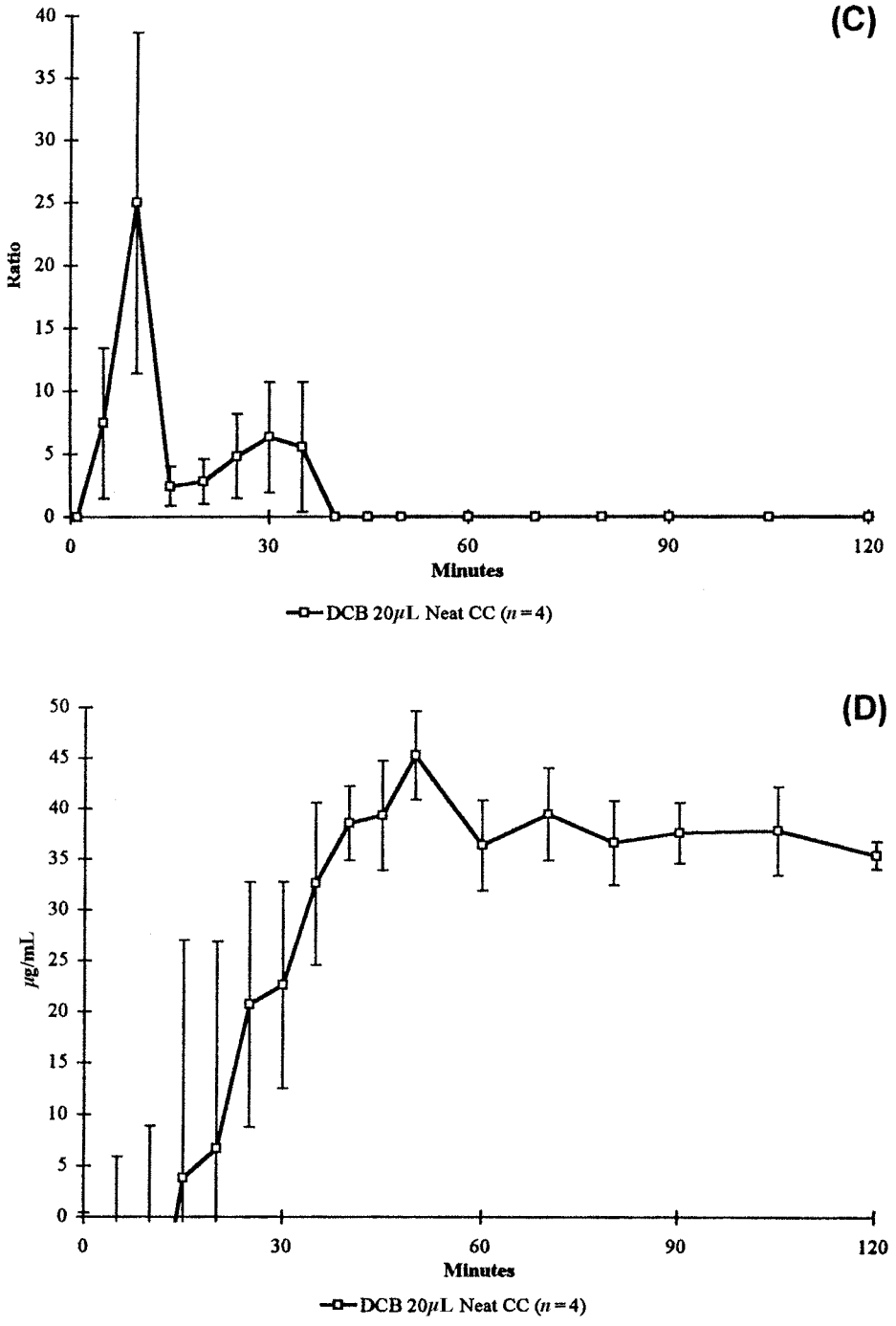

FIGURE 4. (Continued) C. Ratio of DCB in perfusate to vapor. D. Corrected perfusate concentrations using equation. CC, cradle chamber.

DCB beaded on the skin surface, making it impossible to achieve uniform dosing. Even with CPFB, the cylindrical geometry of the IPPSF promoted run-off onto dosing templates.

To address these concerns, the glass dosing dome was utilized. In these cases, neat compound never touched the skin surface; instead, vapor from the applied dose uniformly exposed the skin under the dome. Figures 5A and 6A illustrate the absorption of CPFB (scenario J) and DCB (scenarios K, L, M, and N), respectively, through the skin flap from the glass dosing dome. Figures 5B and 6B compare the dosing dome profiles to the corrected cradle chamber profiles previously presented. The glass dosing dome traps the vapor next to the skin flap, so there is no vapor in contact with the perfusate in the cradle. Notice in Figure 6A that there has been no depletion of the DCB dose, as no defined peak has been demonstrated. This is in contrast to the liquid dosing scenarios in which

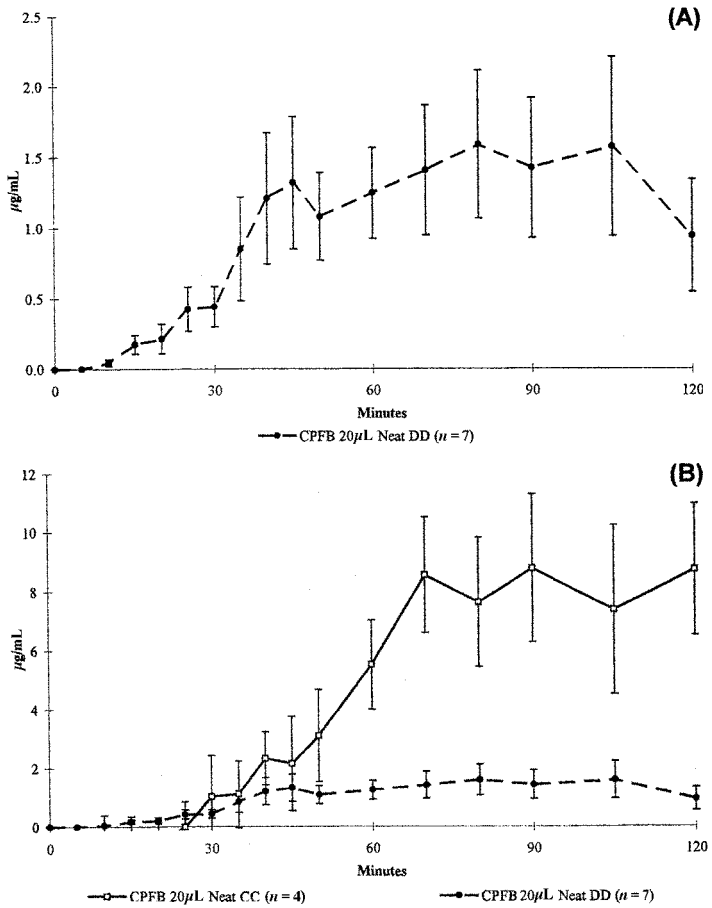

FIGURE 5. A. Percutaneous absorption of neat CPFB dosed in dosing dome. B. Comparison with corrected cradle chamber absorption. DD, dosing dome; CC, cradle chamber.

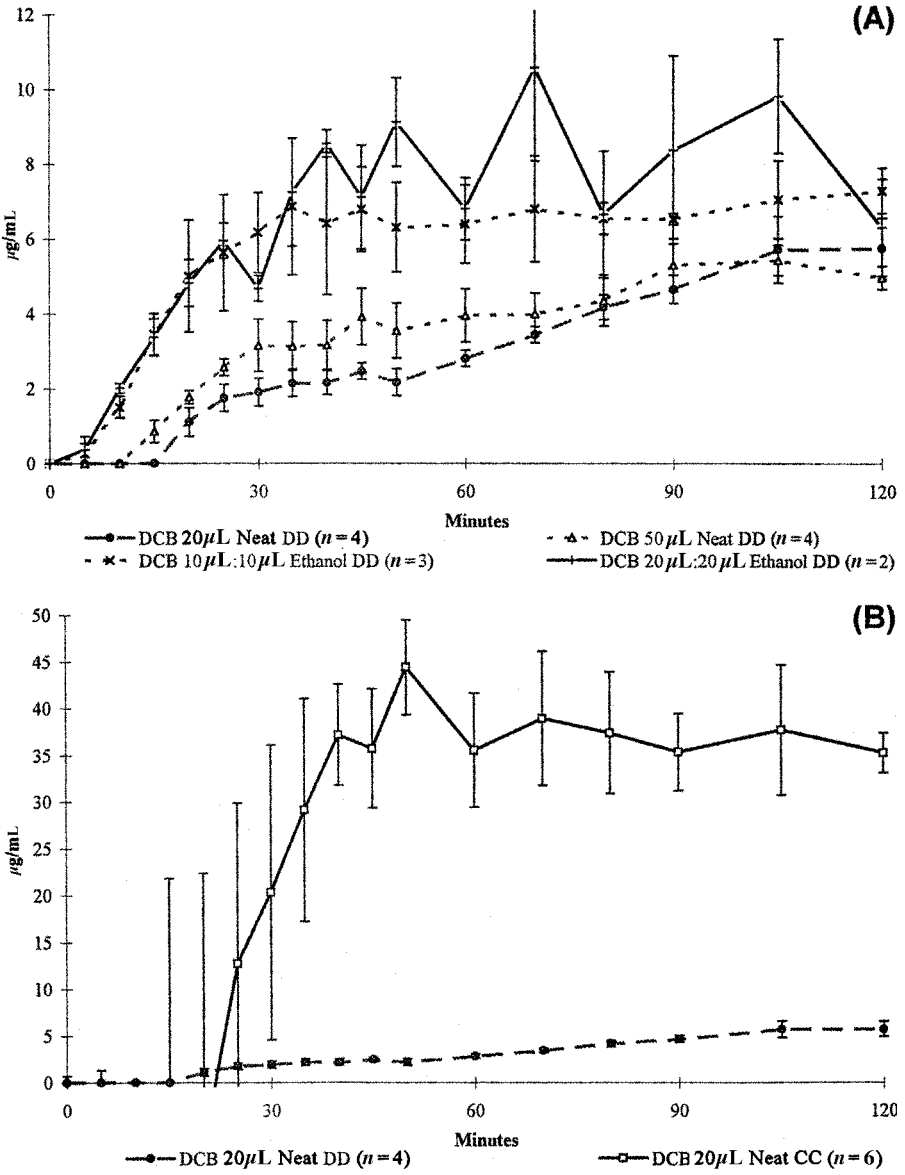

FIGURE 6. A. Percutaneous absorption of DCB dosed according to four treatments: 20 µL neat; 50 µL neat; 10 µL DCB and 10 µL ethanol; 20 µL DCB and 20 µL ethanol. B. Comparison of absorption of 20 µL neat DCB in dosing dome and in corrected cradle chamber. DD, dosing dome; CC, cradle chamber.

evaporation of applied dose depletes available compound for absorption. It is important to note that for both compounds, higher absorption is seen with the liquid cradle chamber dosing than with the vapor dosing dome.

Figure 6A and B depicts the absorption profiles seen with dosing scenarios K (20 µL neat), L (50 µL neat), M (10 µL vapor/10 µL ethanol), and

N (20 μL vapor/20 μL ethanol). With DCB, there is very little dose effect suggesting saturation of absorption. In contrast, dosing with ethanol significantly increased absorption across both doses, suggesting an enhanced permeability of skin in the presence of ethanol vapor.

CONCLUSION

These studies clearly illustrate the complexities involved in assessing the absorption of volatile chemicals across skin. The problems are magnified in a model system such as the IPPSF which imposes restrictions on the type of dosing devices that can be applied to the skin without causing adverse biological effects such as vascular constriction. The initial approach of using the cradle chamber results in absorption data that is confounded by vapor absorption into perfusate. Correction for this effect is possible and reduces some variability; however, it eliminates early time points that may be important for some types of mathematical modeling.

The dosing dome is a simpler approach to this problem, but it allows the assessing of absorption from the vapor phase only. The downside of this technique of experimentation is that a number of domes must be fabricated to fit the different sizes of IPPSFs that are produced from the surgical procedure.

The focus of this work was to compare various dosing methodologies suitable for the IPPSF, not to focus on the mechanism of percutaneous absorption or on its quantification. However, some observations are worth summarizing. As seen with both CPFB and DCB, saturation of absorption is seen when high and low doses are compared. When expressed as percent dose absorbed, the lower doses had higher fractional absorptions. This observation, also seen with many other compounds, must be taken into consideration when such experimental data are utilized in risk assessment calculations. Second, absorption from liquid is not directly comparable with absorption during a vapor phase. Part of this effect could be secondary to the physical chemistry of diffusion, where the driving force in a liquid application is the concentration on the skin surface versus the partial pressure of the compound while it is in a vapor state.

Finally, simultaneous dosing with a vehicle may modulate the absorption profile seen. In the case of DCB in ethanol, higher absorption was seen at both doses when compared to neat DCB application. This confirms the previously alluded to effects of application vehicle (Brooks and Riviere, 1996; Löf and Johanson, 1998). However, all previous studies were done using liquid dosing; thus, this finding is significant because vapor/vapor exposure also results in modulation of absorption.

The mechanism behind vehicle effects is often suggested to be secondary to formulation factors such as solubility in the vehicle and partitioning of the vehicle between the skin surface and the stratum corneum. Finding a vehicle effect when both compounds are in the vapor phase is significant because solution interactions are not present. This suggests that the mechanism is more likely to be secondary to direct interaction of the vehicle with stratum corneum lipids, which results in altered permeability for the penetrating chemical. This observation is important when the absorption of complex mixtures routinely encountered in occupational and environmental exposures is being assessed.

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