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An *In Vitro* Evaluation of Human Dermal Exposure to Benzene Sulfonate, m-Benzene Disulfonate and p-Phenol Sulfonate

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Three benzene sulfonate compounds (benzene sulfonate, BSA; m-benzene disulfonate, BDSA; p-phenol sulfonate, PSA) were identified in groundwater samples taken near a former disposal site (Tay et al., 2004). One or more of the compounds were reported to be present in water sampled from area wells, with levels ranging from <0.005 to 475 mg/L. These compounds are dissociated (as the sulfonate ions) in dilute solution and are not expected, based on the known behavior of other ionized compounds, to penetrate human skin. Given that these compounds were determined to be present in water used by local residents for washing and bathing, it was decided to directly test the compounds to determine whether these compounds penetrate human skin.

An extensive search of the peer-reviewed literature and computer-based bibliographic databases (e.g., Lewis, 1999; Verschueren, 1996; Medline and Toxline databases) revealed no dermal penetration studies or absorption data for BSA, BDSA or PSA. Data from preliminary rabbit bioassays (Tay *et al.*, 2003) revealed that these compounds were not irritating to skin when tested at concentrations that exceeded (>10X) those observed in groundwater.

Skin absorption for the vast majority of compounds is a passive process. Dermal penetration studies undertaken using appropriate *in vitro* experimental conditions have produced data for a wide range of chemicals that demonstrate the usefulness of the method (Bronaugh et al. 1982; Bronaugh and Maibach 1999; Diembeck et al. 1999; Howes et al. 1996; Schaefer and Redelmeier 1996). Standardized protocols for testing the dermal penetration of chemicals have been established by the US Environmental Protection Agency (USEPA 1999a) and the Organization for Economic Cooperation and Development (OECD 2002). These protocols employ standard diffusion cells containing excised human skin, which allow chemicals that are able to penetrate the dermis to be collected and analyzed. In the present study, *in vitro* dermal penetration experiments are carried out to estimate the dermal penetration properties (flux rates) of BSA, BDSA and PSA in aqueous solution. The experimentally measured dermal fluxes (J) and permeability coefficients (K_p) can then be used to calculate the dermally absorbed dose (DAD), an essential component in the calculation of the chronic daily intake

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for quantitative human health risk associated with exposure to water containing benzene.

In vivo, chemicals permeate the skin's diffusion barriers and enter the systemic circulation via capillaries at the dermo-epidermal junction. Percutaneous absorption can be regarded as the translocation of surface-applied chemicals through the various strata of the epidermis and a small portion of the underlying dermis that contains papillary capillaries (where penetrating substances are first delivered to the blood stream). Dermal flux values are quantitative measures of the rate of systemic uptake of chemical via the dermal route. Percutaneous absorption begins with diffusion through the non-viable stratum corneum (SC). Diffusion through the SC is the rate-limiting step in the percutaneous absorption process for the vast majority of chemicals. Fick's first law of diffusion is used to relate the flux (J) of a chemical through the skin under "infinite dose" (i.e., concentration differential across the membrane is infinite and unchanging over time), steady-state conditions:

$$J = D K_p C/h$$

Where: D = effective diffusion coefficient of solute in stratum corneum
K_p = partition coefficient of solute between skin and vehicle
C = concentration of chemical in vehicle
H = effective diffusion path length through the skin barrier

Skin/vehicle partition coefficients are not easy to measure independently, and K_p and D are normally lumped together experimentally to give the apparent permeability coefficient (K_p) as measured in *in vitro* experiments. Flux is directly proportional to chemical concentration and the permeability coefficient. Assessing potential systemic toxicity of human dermal exposures requires having the ability to experimentally measure dermal absorption in the laboratory. A Franz-type diffusion cell is used to measure the absorption of BSA, BDSA and PSA using excised human cadaver skin. In this experimental setup, diffusion of chemical through skin is measured over time by periodically withdrawing a small amount of receptor solution (via the diffusion cell side arm) and determining chemical concentration. Dermal flux is determined by calculating the slope of cumulatively absorbed mass over time.

The purpose of this study was to assess one of several possible exposure pathways, in this case dermal penetration and absorption.

MATERIALS AND METHODS

Sodium benzene sulfonate (CAS# 515-42-4) was purchased from Sigma-Aldrich (14,728-1; Lot # DU 11227LN). Disodium benzene m-disulfonate (CAS# 831-59-4; Lot # G6341A) and 4-hydroxybenzene sulfonate, sodium salt dihydrate

(CAS# 825-90-1; Lot # G9439A) were purchased ABCR GmbH & Co., (Germany), Tetrabutylammonium bromide (CAS# 1643-19-2; 99% pure, Lot # 86857) was purchased from Fluka. Nominal 2 mg/ml solutions of each test material were prepared in deionized water just prior to the start of the dermal study. The measured pH of all three solutions was 6. The measured pH of the deionized water was 6.

Experiments were performed using abdominal skin from one male and two female human cadavers (age range: 35-70 years) procured from the National Disease Research Interchange (NDRI) in Philadelphia, PA. Whole skin sections were stored frozen. Skin sections were shaved (as necessary) prior to using a dermatome (Padgett, Kansas, MO) equipped with a No. 252 blade to slice skin sections (epidermis side) to approximately 350 μm. Prepared skin sections were vertically cut into ~3 x 3 cm sections and then measured with a pressure sensitive micrometer to ensure each section was of a uniform thickness. Each section was then mounted over a 15-mm diameter Franz diffusion cell body and a Teflon o-ring and cell cap placed atop the skin and secured with an adjustable clamp. Each experimental group (n=6 cells/test material) contained tissue from three different human subjects.

The diffusion cells were contained in a Franz diffusion unit (Crown Glass, Somerville, NJ). A saline solution (0.9%) was used as receptor fluid. Temperature of the diffusion cells was maintained at 37°C by attaching the water-jacketed cells to circulating water baths. Integrity of the human skin sections was evaluated with ^{3}H-water before use; only sections exhibiting normal water permeation (K_p<1.0 x 10-2 cm/hr) were used for studies. Mounted skin sections were dosed at exactly 1.0 ml of dosing solution/diffusion cell for the 24 hr experiment (n=6 cells/test article). More specifically, cell numbers 1 through 6, 7 through 12 and 13 through 18 were dosed with BSA, BDSA and PSA, respectively. Receptor fluid was sampled (400 μl) at 8, 16 and 24 hr post-dose.

Radioactivity (^{3}H-water) in the receptor fluid from the tissue integrity pre-study was quantified using a Beckman LS 5801 liquid scintillation counter. Receptor fluid aliquots collected during the study were analyzed for the presence of test materials with an HPLC system consisting of a Beckman Model 420 Solvent Programmer, Model 507 autosampler/injector and Model 166 Variable Wavelength Detector. A Phenomenex Luna 5μM Phenyl-Hexyl, 250 x 4.6-mm column (serial no. 73455-7) was used. The eluents were prepared from Fisher HPLC-grade methanol, deionized water and tetrabutylammonium bromide (TBAB). BSA, BDSA and PSA were analyzed using an isocratic mobile phase of 53% 25-mM aqueous TBAB: 47% methanol monitoring absorption at 263, 267 and 271 nm, respectively. The flow rate was set at 1.0 ml/min and the injection volume was 50 μL.

Separate stock solutions containing all three benzene sulfonates were prepared at nominal concentrations of 1, 0.5, 0.2, 0.1 and 0.05 mg/ml in 0.9% saline. The initial standard curve was prepared by adding 10 µl of the sulfonate stock solutions to 640 µl of 0.9% saline and 350 µl of 75 mM TBAB solution, resulting in nominal HPLC standard concentrations of 10, 5, 2, 1 and 0.5 ng/µl of each of the three sulfonates. All three initial calibration curves had linear regression coefficients (r) >0.99 and mean calibration factor (CF) relative standard deviations (RSD) less than 20%, thereby allowing the use of the linear model (USEPA, 1999b), and the mean CF values to determine sample concentrations. The initial calibration curves were evaluated at each sampling time point over the course of the dermal experiment (using the 1 ng/µl standard for each test material) and found to be within the ±15% (USEPA, 1999b) verification guideline. The lowest concentration standard in the five-point standard curves (0.5 ng/µl) were designated as the estimated quantitation limits (EQLs) for the three sulfonates based on a review of the signal to noise ratio of the standard prepared in the matrix blank. Each peak for each individual standard could be clearly distinguished from one another when scanned at different wavelengths, with respective elution times of 9.8, 16.0 and 5.8 minutes.

RESULTS AND DISCUSSION

The experimental procedures used in this study adhere to the general guidelines for conduct of in vitro dermal absorption studies (USEPA 1999a,b; OECD 2002). Key components of the experimental procedure are 1) the use of non-viable human tissue, 2) evaluation of tissue barrier integrity using tritiated water and 3) use of tissue from multiple subjects within each test group. The present study used skin sections from four subjects; each test group contained tissue from at least three subjects. The tritiated water Kp-values were all measured at ≤4 x 10-3 cm/hr for tissue samples in the three test groups.

With the exception of diffusion cell #18 in the cell #13 - 18 group (PSA), none of the test materials were detected in any of the diffusion cells at the method EQL of 0.5 ng/µl through 24 hrs. Figure 1 shows a typical 24-hr receptor fluid chromatogram for BSA overlaid with a standard run chromatogram of all three standards in a matrix blank with BSA quantitated at 263 nm. Seventeen out of eighteen cells gave results similar to this, with no evidence of the presence of the test chemicals in the receptor fluid at 8, 16, or 24 hrs post dosing. Cell #18, however, did show the presence of BSA in the receptor fluid. The data for cell #18 can be demonstrated statistically to be an outlier. A review of the experimental data shows: 1) tissue from the same donor was used from cell #17 and #18 and no PSA was detected in the receptor fluid of cell #17 and 2) tissue from two other donors was used for cells #13 - 16 and no PSA was detected in the receptor fluid of these cells. Thus, it is likely that there was post-dosing barrier

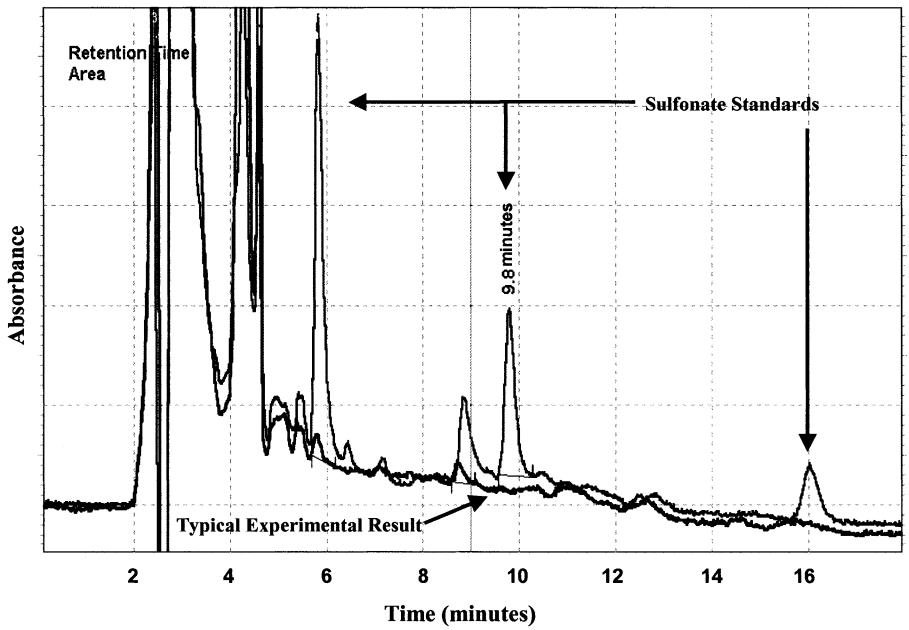

Figure 1. Three sulfonate standards (1 ng each) in matrix blank (top, with BSA retention time noted) and a typical 24-hr benzene sulfonate experimental results (bottom).

function failure in the skin tissue in cell #18. In addition, the flux of PSA through the skin in cell #18 was not constant over the three time points measured (8, 16 and 24 hrs). The flux at the three time points was 1137, 1241 and 1495 ng/cm2/hr. These changing flux rates are not consistent with a uniform skin penetration of the test substance, but appear to be more consistent with a barrier function failure which was becoming more marked with time.

To ensure that the absence of test materials in the receptor fluid of the test cells did not reflect an unexplained loss or degradation of the test materials, recovery experiments were undertaken. At the end of the experiment, test solution from the top of the skin surface was recovered and analyzed. In the case of BSA, the average recovery was 107%. Average recovery was 97% for BDSA and average recovery of PSA was 85%. This demonstrates that the test articles were available for penetration through the skin, and the absence of the materials in the receptor fluid was explained by the fact that the materials did not penetrate the skin. Although the estimated quantitation limit (EQL) for the three sulfonates may well have been lower than the 0.5 ng/µl standard, the EQL for the study was defined as the concentration of the lowest non-zero standard in the calibration curve (0.5

ng/μl). In the absence of detectable amounts of analyte in the test cell receptor fluids through 24 hrs, plausible estimated permeability constants (K_p) for all three test materials (based on one-half the EQL (0.25 ng/μl) would be $K_p = 2 \times 10^{-5}$ cm/hr.

In summary, based on a standard *in vitro* skin absorption method endorsed by both the USEPA and the OECD, BSA, BDSA and PSA do not appear to penetrate human skin. The results are not surprising in lieu of the fact that charged polar molecules, in this case completely dissociated organic acids, are not generally known to be physiologically capable of penetrating the comparatively lipophilic stratum corneum. The K_p's that were estimated, based on one-half of the EQL for all three compounds, were ~100 times lower than that measured for deionized water (2×10^{-3} cm/hr). Human exposure via the dermal route would therefore be expected to be insignificant, as would any subsequent risk.

The experimental results strongly support a fundamental principle in the field of toxicology: namely, the observation that ionized compounds do not generally present a significant dermal risk to humans because they are not easily absorbed through skin and thus are not able to enter and target any living cell, tissue or organ system. We conclude that, should residents use well water containing one or more of the aforementioned benzene sulfonate compounds for bathing or washing, the possibility of dermal absorption is insignificant. The attendant risk would therefore also be, for all practical purposes, immeasurably low.

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