Improving the Sensitivity of In Vitro Skin Penetration Experiments

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The institution of a readily-implemented sample screening and data handling procedure for in vitro skin penetration studies yields substantial improvements in sensitivity for distinguishing between formulations, treatments, penetrants, etc. The procedure involves four steps: 1) prescreen the tissue samples to determine their intrinsic permeability; 2) apply treatments using a randomized complete block (RCB) design, with blocking by tissue permeability; 3) apply a variance-stabilizing transformation to the penetration data, followed by outlier testing; and 4) analyze the transformed data according to an RCB analysis of variance, using tissue permeability as the blocking variable. For penetration studies in which high sample variability is a concern, the above procedure commonly yields a sensitivity advantage of several-fold versus alternative methods of comparison.

KEY WORDS: in vitro skin penetration; human cadaver skin; statistics; data transformations; data analysis; sensitivity.

INTRODUCTION

Common objectives of in vitro skin penetration studies include the selection of topical or transdermal drug formulations, dermal exposure assessment for environmental toxins, and estimation of the harshness of skin care products via their effect on skin barrier function. The importance of choosing the right model system for such studies (e.g., skin species and thickness, dose, degree of occlusion, and receptor phase composition) in order to obtain relevant results has been well documented (1–7). This paper focuses on a subsequent question: Having chosen the best available model system for a penetration study, how can one maximize the sensitivity of the test for distinguishing between treatments? This question is particularly important when working with a highly variable substrate such as human cadaver skin which, despite its problems, is often the model of choice (5–7). Implementation of the procedure described herein gives up to three-fold higher sensitivity for distinguishing between treatments in a single study. Furthermore, the tissue screening aspect can lead also to greater reproducibility of skin penetration values between studies.

To show why the proposed experimental design and analysis can significantly impact results, we first consider the nature of cadaver skin penetration data. The complex structure of skin with its multiple diffusion pathways and (in vitro) its varying thickness and degree of damage leads to a distribution of experimental penetration rates that is a function of exposure time and of the physicochemical properties of the test compound.

Ionic compounds yield broad and highly right-skewed permeability distributions in skin (8–10). Freshly excised tissue yields less variable penetration data than does frozen tissue (10). Our own experience extends these observations to neutral but poorly lipid-soluble compounds that might be expected to penetrate skin via polar pathways (GBK, unpublished data). These facts alone lead us to associate the right-skewed nature of the permeability distributions for these materials with microscopic damage incurred during tissue collection and storage. The transport of ions and water-soluble compounds that cannot easily penetrate the stratum cornueum lipid bilayers would be expected to be highly sensitive to the number of defects present in the lipid barrier.

Figures 1 and 2 present evidence that the permeability distributions for human cadaver skin to other agents, both hydrophilic and lipophilic, are also highly skewed. In many cases they can reasonably be taken to be lognormal, as shown in the insets. Figure 1a shows a typical distribution of water penetration values through samples of excised human cadaver skin obtained from a single donor. The results were obtained using the 3H2O penetration test developed by Franz and Leher (11) and described in the Experimental section. The tissue had been harvested at autopsy by standard methods and stored frozen (9). Other skin samples identically prepared have had either narrower, more sharply peaked distributions or broader, relatively flat distributions. In our experience with this test, the primary difference from study to study lies not in the mode of the distribution (usually about 0.3–0.8 μL/cm²), but rather in the percentage of high permeability samples. In other words, the permeability distributions differ in the weight of the right-skewed tail. We have observed that freshly excised skin (either human surgical waste or animal skin) tends to have a narrower, more symmetrical permeability distribution than does skin bank skin. However, the highly skewed distribution of cadaver skin water permeability values obtained over a large number of studies is clearly shown in Fig. 1b.

The data in Fig. 1b are skewed even if highly permeable samples are excluded from the analysis. In fact, a good fit to the lognormal (Shapiro-Wilk W = 0.981, p = 0.157) is obtained even after excluding samples whose penetration values exceed Franz and Leher’s acceptance criterion of 1.2 μL/cm².

Figure 2 shows that a lognormal distribution may also be obtained with lipophilic compounds. This figure shows the distribution of pooled penetration data from seven cadaver skin penetration studies involving 36 different permeants (12). The data for each compound have been normalized by dividing by the median in order to remove differences related to the center of the distributions and leave only information related to shape. The linearity of the lognormal probability plot and coincidence of the 50th percentile with the median demonstrate how well the lognormal describes the pooled data. Shapiro-Wilk testing supports this conclusion (W = 0.983, p = 0.465).

The test permeants in Fig. 2 were predominately small, lipophilic compounds, although they included several weak bases dosed as salts. Most of the latter compounds probably diffused through the skin in their neutral form due to pH-partition equilibria; in any case, their permeability distribu-

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Fig. 1. Frequency distribution of water penetration values through split-thickness human cadaver skin using the $^3H_2O$ penetration test described in the text. (a) Results from 66 samples derived from a single donor. (b) Results from 539 samples derived from 7 donors. The inset shows a lognormal probability plot of the distribution.

Fig. 2. Frequency distribution of steady state penetration rates of assorted drugs and other lipophilic compounds through split-thickness human cadaver skin about the median value for each compound. The data are taken from Ref. 12. The plot shows results from 3-4 donors and 302 individual samples (242 samples after excluding the medians). The inset shows the lognormal probability plot. The compounds studied were acetaminophen, benzoic acid, benzyl alcohol, caffeine, clonidine hydrochloride, dextromethorphan, dextromethorphan hydrobromide, diazepam, estradiol, ethacrynic acid, 5-fluorouracil, furosemide, griseofulvin, hydralazine hydrochloride, hydrocortisone, ibuprofen, indolyl-3-acetic acid, indomethacin, isosorbide dinitrate, ketoprofen, methyl salicylate, minoxidil, mor- phine sulfate, naproxen, nicotinic acid, nifedipine, pentazocine, pentazocine hydrochloride, piroxicam, propranolol hydrochloride, salicylamide, salicylic acid, sulindac, terbutaline sulfate, testosterone, and trimcinolone acetate.

mous "blocks." Random assignment of treatment replicates into each of these blocks is called a Randomized Complete Block (RCB) design. The subsequent analysis of variance of this design (RCB ANOVA) is potentially much more precise than a standard ANOVA. The RCB analysis compares treatments within blocks and then pooled these comparisons over blocks. This takes the inherent block-to-block tissue variability out of the analysis and, in essence, compares treatments on more homogeneous tissues. Thus one can actually use knowledge of these inherent differences in tissue sample permeability to one's advantage, by designing and analyzing such data appropriately (i.e., RCB ANOVA) so that the most precise treatment comparisons can be made. Otherwise, if a simple ANOVA is performed, the block-to-block tissue variability is not partitioned out and inflates the standard error of the treatment comparisons, yielding less precise comparisons.

The third procedure involves the mathematical transformation of penetration values prior to analysis, followed by outlier testing, so as to obtain more nearly normally distributed, equal variance responses. The transformation step can be accomplished by either taking the logarithm or (more generally, as described in Appendix 1) by applying a quasi-logarithmic transformation. Outlier testing and elimination can then be conducted using standard methods (13,14). This procedure allows common parametric statistical tests to be appropriately applied to the data; hence, valid conclusions can be drawn regarding treatment comparisons. The combi-