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On the non-Gaussian distribution of human skin permeabilities

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Summary

Human skin demonstrates considerable inter- and intra-donor variability to transdermal drug permeation. In the past, for a given permeant, replicate measurements of diffusional parameters have been (at least tacitly) assumed to provide a normal distribution with a wide degree of dispersion. We have monitored the permeability of human abdominal skin in vitro to model hydrophilic (5-fluorouracil, 5-FU) and lipophilic (oestradiol, ES) drugs. Drug permeation from a saturated aqueous solution was assessed using stainless-steel diffusion cells with an aqueous flow-through receptor solution. Diffusion was characterised by evaluation of drug permeability coefficients at steady-state flux. The variability of drug permeability coefficients for 644 determinations of 5-FU from 71 skin specimens and 221 replicates of ES from 28 specimens did not follow a normal distribution. Our data show that permeability coefficients for both drugs followed more closely log-normal distributions. Additionally, our results indicate that the specimen population of cadavers may be comprised of two subgroups; those with higher than expected permeability, and the majority providing 'ordinary' permeability. No relationship was evident between specimen age, sex or storage time and drug permeability coefficient. When data are found to be log-normally distributed the appropriate measure of central tendency is the geometric mean, since the arithmetic mean tends to be artificially increased because of the nature and degree of a skewed distribution.

Introduction

The variable nature of human skin to transdermal drug permeation is well documented. Site-to-site differences within the same donor in percutaneous absorption have been attributed to many factors including variations in stratum corneum structure, thickness and lipid content, and dissimilarities between local metabolism and microcirculation at various body regions (Feldman and Maibach, 1967; Scheuplein and Blank, 1971; Elias et al., 1981; Bennett and Barry, 1986). Additionally, for a given body site, variations exist between different specimens (Michaels et al., 1975); typically, for chemicals such as octanol and caffeine permeating in vitro through human skin, inter-sample deviations provide coefficients of variation of approx. 40% to diffusional parameters (Southwell et al., 1984). A high degree of intersubject variability has also been noted in vivo; the doses of nitroglycerin delivered from transdermal devices showed coefficients of variation of between 30 and 40% among different individuals (Noonan and Gonzalez, 1990).

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Intra-sample variations (differences within a given specimen) have also been reported. Differences in diffusional parameters are generally somewhat less within a sample than between different samples with intra-specimen coefficients of variation typically being 20–30% (Southwell et al., 1984). However, such variability is a significant problem in percutaneous absorption studies dictating that the determination of the flux of a permeant through human skin from a single source may be accurate only to within approx. 30% (Kastings et al., 1987).

In studies examining the modes of action of 33 terpenes, terpenoids and essential oils as penetration enhancers, including unpublished experiments, we monitored the permeability of human abdominal skin in vitro to model hydrophilic (5-fluorouracil, 5-FU) and lipophilic (oestradiol, ES) drugs (Williams and Barry, 1989; 1991a,b). The basic experimental protocol concentrated on determining enhancement ratios, i.e., the quotient of the permeability coefficient determined after enhancer treatment to that before such a procedure. On re-examination of our accumulated raw data, we have now observed that the permeability coefficients of the two permeants through the untreated membranes did not follow a normal distribution. The present paper considers this observation in more detail.

Materials and Methods

The experimental protocol was as previously described (Williams and Barry, 1989; 1991a,b), hence only brief details are presented here.

Preparation of human epidermal membranes

Caucasian mid-line abdominal skin samples were obtained post-mortem and were stored frozen at −20°C (Harrison et al., 1984). Samples used were both male and female (37:63%) and ranged from 17 to 89 years with a mean of 70 years.

Epidermal membranes were prepared by the heat separation technique of Kligman and Christophers (1963). Excess fatty and connective tissues were removed from the skin which was then immersed in 60°C water for 45 s. The epidermal membrane was teased off the underlying dermis and floated on an aqueous solution of sodium azide for 36 h to ensure that the stratum corneum was essentially fully hydrated.

Permeants

The model hydrophilic permeant was 5-[6-^{3}H]fluorouracil (Amersham International plc), a saturated aqueous solution being prepared with the help of unlabelled 5-FU (Sigma Chemical Co.). [2,4,6,7-^{3}H(N)]Oestradiol (NEN Research Products) was the test lipophilic permeant. Unlabelled oestradiol (Sigma Chemical Co.) was used to prepare a saturated aqueous drug solution.

Permeation experiments

Experiments at 32 ± 1°C used an automated diffusion apparatus with 24 stainless-steel diffusion cells, diffusional area 0.126 cm^2, and 0.002% aqueous sodium azide as flow-through receptor solution (Akhter et al., 1984). Fully hydrated epidermal membrane samples were mounted in the cells and 150 μl aliquots of a saturated, radiolabelled drug solution were placed in the donor compartments which were covered. For 5-FU, 4 ml samples of receptor solution were collected every 2 h for 36 h; 5-FU has a relatively high aqueous solubility and low permeability coefficient, and donor depletion of the drug during a permeation experiment is negligible (generally less than 2%). However, oestradiol has a relatively low aqueous solubility and high permeability coefficient resulting in possible donor depletion. Hence, for ES, a crystal of the drug, with the same radioactivity as the donor solution, was placed in each donor compartment and the donor drug solution was replenished every 8 h to ensure a saturated ES solution (Williams and Barry, 1991b). For the steroid, samples (2 ml) of receptor fluid were collected every hour for 24 h. Both radiolabelled drugs were determined by liquid scintillation counting (Packard 460C). Linear regression analysis of the pseudo steady-state diffusion results allows evaluation of the permeability coefficient (K_p) for the drug in the membrane.

Results and Discussion

The arithmetic relative frequency distributions of permeability coefficients through human skin in vitro for 644 determinations of 5-FU from 71 skin specimens and 221 replicates of ES from 28 specimens are in Fig. 1.

Clearly, the data in Fig. 1 show skewed distributions. Logarithmic transformations of the data are illustrated in Fig. 2, demonstrating potential bimodal near log-normal frequency distributions.

Figs 1 and 2 provide the raw experimental values from our studies. Typically, we obtain eight values for drug permeability coefficients per cadaver specimen. However, during a diffusion study a sample of epidermal membrane may fail through, for example, tissue damage. In such an event, the permeability coefficient is rejected and omitted from our analysis. Hence, while representing events at a practical level, the data in Figs 1 and 2 may be numerically biased as all specimens do not have the same number of replicates.

To avoid the problems of biased results, the geometric mean drug permeability coefficient per cadaver was calculated and the data are presented in Fig. 3 as relative frequency distributions (on an arithmetic scale). Evidently, the mean permeability coefficients for both drugs show skewed distributions. Additionally, Fig. 3 indicates that some cadavers provide higher than expected permeability coefficients, i.e., the population may possibly contain two subgroups, one subgroup comprising specimens providing higher than the expected 'ordinary' values. Clearly this possibility could be further investigated with greater numbers of replicates. The data presented here result from work over the last 4 years involving two experimentalists and approx. 100 cadaver specimens. With continuing studies we hope to add to this large database to investigate the possibility of population subgroups.

A logarithmic transformation of the data in Fig. 3 is given in Fig. 4, illustrating that the average permeability coefficients per cadaver are near log-normally distributed for 5-FU although the graphical evidence is less convincing for ES, possibly because of the more modest sample size.

The Kolmogorov-Smirnov test can be applied to the logarithmically transformed data in Fig. 4 to compare the cumulative distribution function of the experimental values with a Gaussian distribution (Fig. 5). For both drugs, the logarithmic distributions show no significant difference ($\alpha = 0.05$) from a Gaussian distribution (for 5-FU,

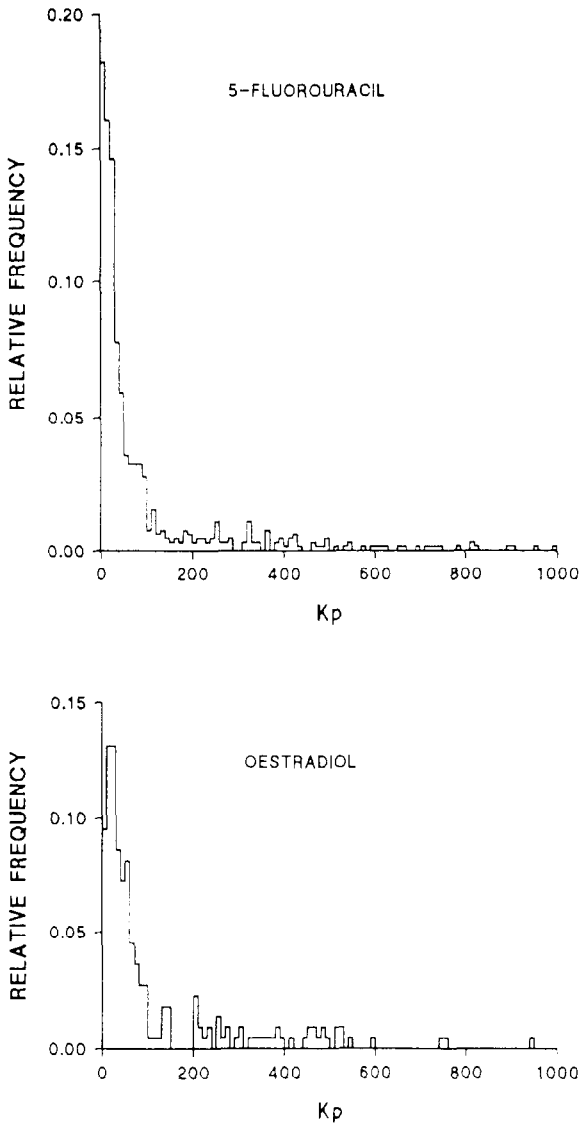

Fig. 1. Relative frequency distributions of aqueous 5-fluorouracil and oestradiol permeability coefficients through human skin in vitro. For 5-fluorouracil $n = 644$, $K_p \times 10^6$ cm/h; for oestradiol $n = 221$, $K_p \times 10^4$ cm/h.

$D_{maximum} = 0.0775 < D_{critical} = 0.1614$, $n = 71$; for ES, $D_{maximum} = 0.1318 < D_{critical} = 0.2570$, $n = 28$).

In order to confirm the nature of drug permeability coefficient distributions, the cumulative frequency of averaged K_p values is plotted on a probit scale against the permeability coefficients on a logarithmic scale (Fig. 6). The linear relationship indicates near log-normality.

Additionally, the Davies test for logarithmic distributions may be applied to the data in Fig. 3

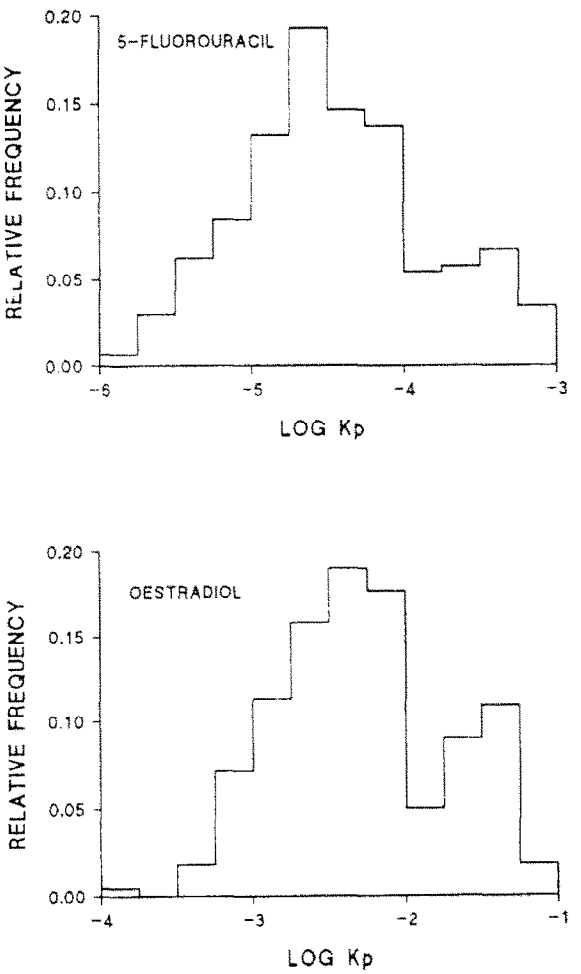

Fig. 2. Relative frequency distributions of logarithmically transformed permeability coefficients for aqueous 5-fluorouracil and oestradiol through human skin in vitro. For 5-fluorouracil $n = 644$; for oestradiol $n = 221$.

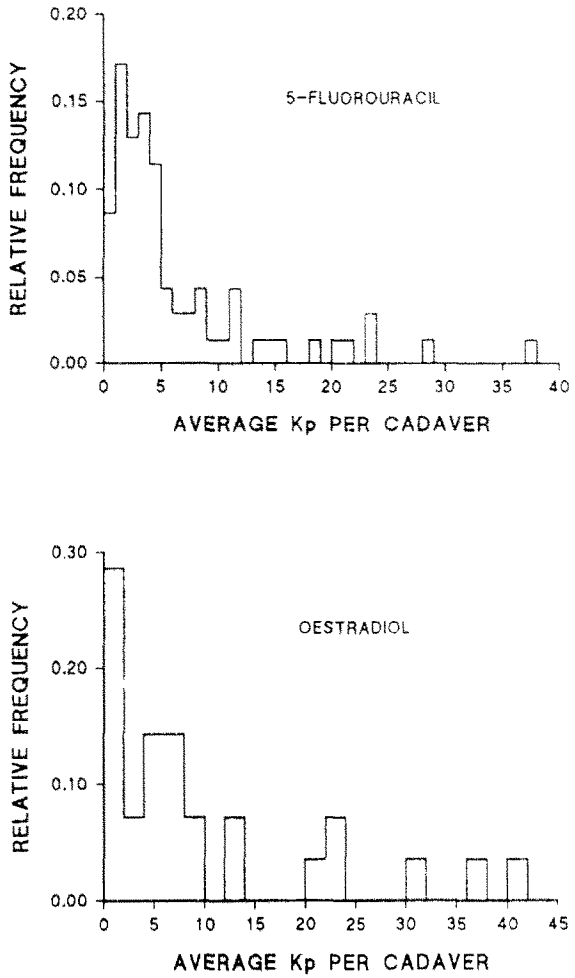

Fig. 3. Relative frequency distributions of the geometric mean permeability coefficients per cadaver for aqueous 5-fluorouracil and oestradiol through human skin in vitro. For 5-fluorouracil $n = 71$, $K_p \times 10^5$ cm/h; for oestradiol $n = 28$, $K_p \times 10^3$ cm/h.

(Langley, 1979). Calculating a 'coefficient of skewness' by:

$$\frac{(\log LQ + \log UQ) - (2 \times \log MQ)}{\log UQ - \log LQ}$$

where LQ is the lower quartile value, MQ the middle quartile value (i.e., median) and UQ the upper quartile value gives values of $+0.17$ and -0.27 for 5-FU and ES, respectively. As both answers are less than $+0.20$, the data are approx-

imately log-normally distributed. It should be noted that ideally this test is more reliable when the sample contains at least 50 observations because quartile values are unreliable with small samples. The ES value quoted above is derived from 28 observations but, when viewed in conjunction with the Kolmogorov-Smirnov test and the probit analysis, this coefficient gives a good indication of a near log-normal distribution.

Intra-cadaver variability provides typical arithmetic coefficients of variation of 15–30%, values

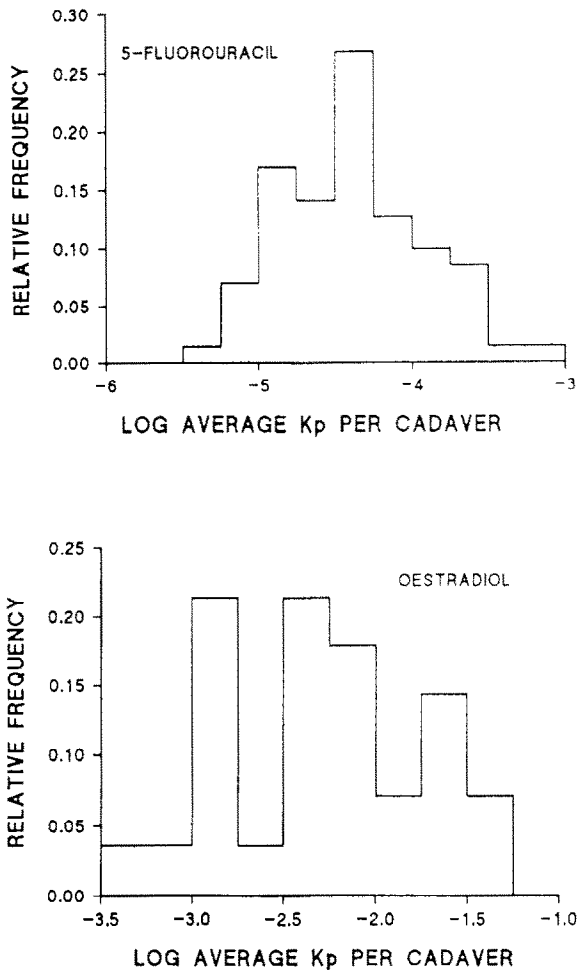

Fig. 4. Relative frequency distributions of logarithmically transformed geometric mean permeability coefficients per cadaver for aqueous 5-fluorouracil and oestradiol through human skin in vitro. For 5-fluorouracil $n = 71$; for oestradiol $n = 28$.

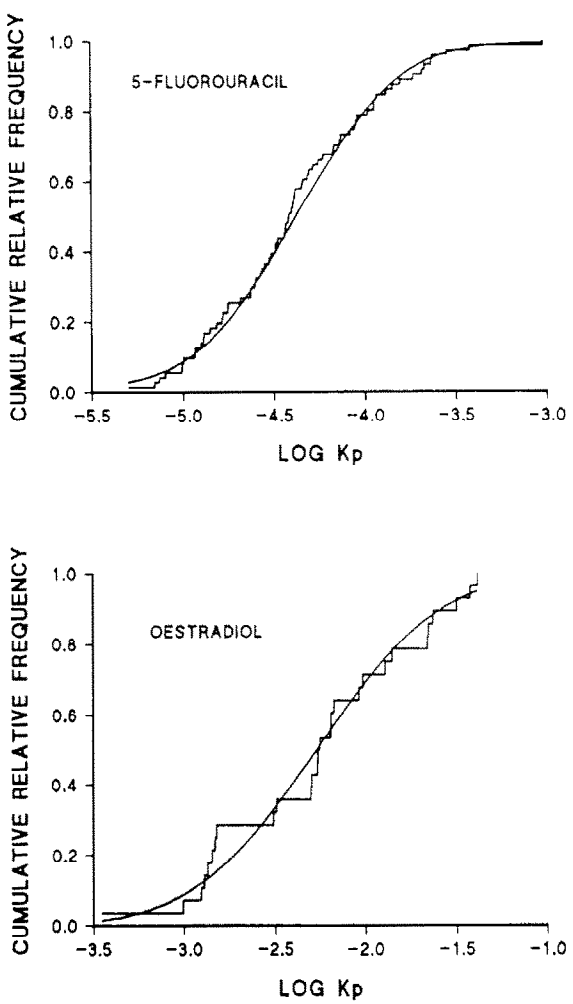

Fig. 5. Cumulative observed frequency distribution of logarithmically transformed geometric mean permeability coefficients per cadaver for aqueous 5-fluorouracil and oestradiol through human skin in vitro (stepped lines). Superimposed are the cumulative expected distributions assuming normality (sigmoid line). For 5-fluorouracil $n = 71$; for oestradiol $n = 28$.

in close agreement with published data (Southwell et al., 1984). Because of the size of our cadaver specimens we can generally obtain a maximum of approximately only 12 epidermal samples per specimen even though we use very small diffusion cells; thus, we have insufficient replicates to determine whether intra-specimen variability is log-normally distributed. However, our data give an indication that this is the case and

no normal (Gaussian) distribution for intra-cadaver variability is evident.

From our experimental data (Figs 1 and 2), comparisons may be made between different average values for the drug permeability coefficients through human skin (Table 1). Averages have been calculated for the two potential subpopulations (lower range using K_p values from log − 6 to log − 3.875 for 5-FU, log − 4 to log − 1.875 for ES, higher range using the remaining data for both drugs) in addition to overall averages for both data sets.

It is widely accepted that human skin provides a highly variable membrane with respect to permeation parameters. However, it is implicit in most literature reports where arithmetic means and standard deviations are quoted that this variability is normally distributed with a wide range. Part of the reason for this untested assumption is probably that in most experiments with human skin, the number of replicates is small, typically of the order of 3–6. Our data for much larger numbers contradict this view. It is our experience that, on the basis of these skin specimens, drug permeability coefficients for 5-FU and ES are closer to being log-normally distributed. Our data also show similarly shaped distributions for the two model drugs (Fig. 2) although, as a lipophilic drug (log $P_{\text{octanol/water}}$ 2.29), ES has a mean permeability coefficient two orders of magnitude greater than that of the hydrophilic drug 5-FU (log P − 0.89). No significant degree of correlation was detected between skin specimen age or sex and drug permeability coefficients.

The existence of bimodal distributions (Figs 2 and 3) may be a real effect with population subgroups providing low and 'normal' skin resistances to drug diffusion: bimodal distributions are also seen, for example, in some pharmacokinetic responses such as the genetically controlled elimination of isoniazid (Rowland and Tozer, 1989). Additionally, a recent conference presentation describing in vivo variability of infrared spectral peaks of human skin, transepidermal water loss, skin impedance and skin surface temperature also noted that skin surface temperature measurements followed a bimodal distribution in that males differed significantly from females (Boddé et al., 1991). However, from our in vitro diffusion experiments, it is possible that the very high values for permeability coefficients may also be due to experimental artefacts. Epidermal membranes are relatively fragile and may be damaged in preparation, and the results from tissues with gross defects are easily rejected during a diffusion experiment. However, preparations with less than catastrophic weaknesses (for example, those with damage around hair follicles when epidermal membranes are teased off the underlying dermis) may not be rejected out-of-hand yet the damaged tissue will provide greater drug fluxes than those obtained through intact

TABLE 1

Average values for the permeability coefficients (K_p) of 5-fluorouracil (5-FU) and oestradiol (ES) through human epidermal membranes at 32 ± 1°C

	Average K_p ± S.D.					
	5-FU ($\times 10^5$) (cm/h)			ES ($\times 10^3$) (cm/h)		
	Lower range [a]	Upper range [b]	All data [c]	Lower range [d]	Upper range [e]	All data [f]
Arithmetic mean	3.29 ± 3.01	38.7 ± 21.2	9.61 ± 15.5	4.03 ± 2.90	36.1 ± 16.6	11.7 ± 16.1
Geometric mean	3.21 ± 2.37	33.2 ± 17.6	3.38 ± 3.82	3.19 ± 2.45	33.2 ± 16.8	5.23 ± 3.92
Median	2.47	33.5	3.15	3.31	33.6	4.81

[a] For lower distribution on Fig. 2; range 0.1–13.3 × 10^{-5} cm/h (log − 6 to log − 3.875), n = 529.
[b] For upper distribution on Fig. 2; range 13.3–100 × 10^{-5} cm/h (log − 3.875 to log − 3), n = 115.
[c] n = 644.
[d] For lower distribution on Fig. 2; range 0.1–13.3 × 10^{-3} cm/h (log − 4 to log − 1.875), n = 168.
[e] For upper distribution on Fig. 2; range 13.3–100 × 10^{-3} cm/h (log − 1.875 to log − 1), n = 53.
[f] n = 221.

tissue. Experimental artefacts may also provide lower than actual drug permeability coefficients if, for example, a small air bubble forms beneath the tissue during a permeation run, small enough to be unobserved but which effectively reduces the cross-sectional area available for diffusion. Such effects may be particularly difficult to see in, for example, opaque diffusion cells. Gross errors due to experimental artefacts are apparent

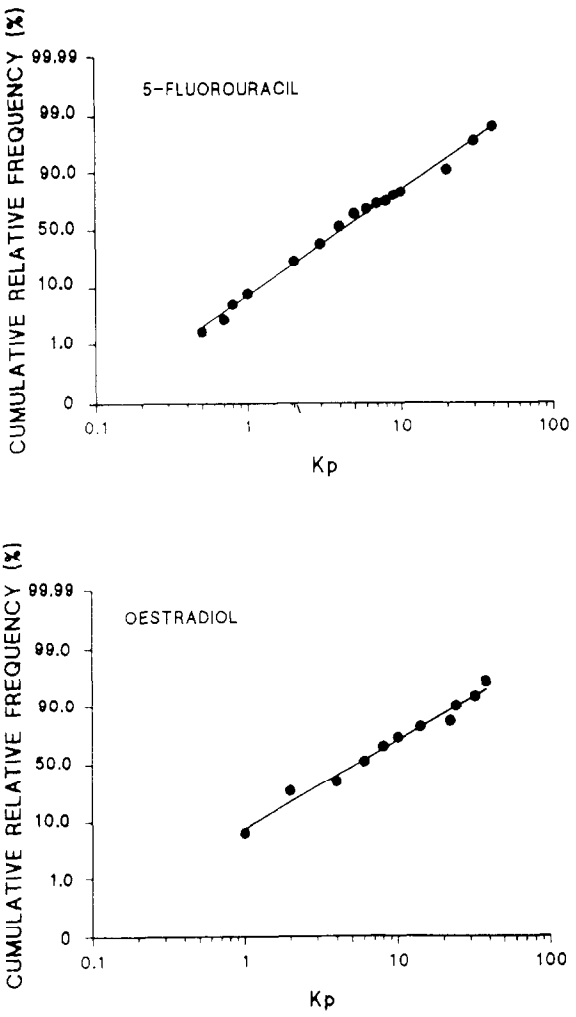

Fig. 6. Probit analyses showing the cumulative relative frequency (on a probit scale) of logarithmically transformed geometric mean permeability coefficients per cadaver for aqueous 5-fluorouracil and oestradiol through human skin in vitro. For 5-fluorouracil $n = 71$; for oestradiol $n = 28$.

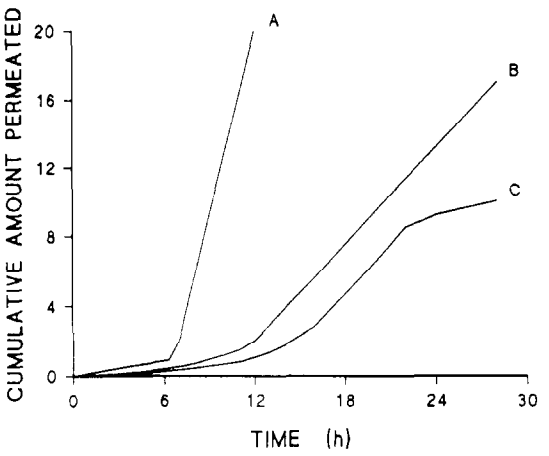

Fig. 7. Diagrammatical representation of drug permeation profiles through human skin in vitro to illustrate experimental artefacts. (A) Gross tissue damage apparent after 6 h; (B) normal permeation profile; (C) air bubble developing under the membrane after 20 h.

from drug permeability profiles, as illustrated in Fig. 7.

Erroneous values arising from leaky tissue or because of the formation of air bubbles may thus be rejected and have not been included in this report. Additional complications arise due to the fact that we have limited control over the treatment which incoming skin samples have received during post-mortem treatment and little account can be taken of the skin donor's history. However, there is no indication that our skin preparation procedures (or storage) affect drug permeability; the flux of oestradiol from a saturated aqueous solution across epidermal membranes was not significantly different to that across full thickness tissue, indicating that our membrane preparation procedures do not introduce gross tissue defects (Williams and Barry, 1991b).

Outliers present a serious problem for statistical analysis and should only be rejected with good cause (Bolton, 1984). In skin permeation research, workers tend to accept data ranging approximately within an order of magnitude from the expected mean result, with occasional higher data beyond this criterion being rejected. If we follow this procedure with the present data we obtain the lower ('expected') log K_p distributions

illustrated in Fig. 2. By rejection of such high data, we obtain arithmetic mean ($\pm$S.D.) values for drug permeability coefficients at 32 ± 1°C of $3.29 \pm 3.01 \times 10^{-5}$ cm/h ($n = 529$) and $4.03 \pm 2.90 \times 10^{-3}$ cm/h ($n = 168$) for 5-FU and ES, respectively, showing good agreement with literature values reported for these hydrophilic (Cohen and Stoughton, 1974; Goodman and Barry, 1988) and lipophilic (Harrison et al., 1984; Flynn and Stewart, 1988; Goodman and Barry, 1988) drugs. If, however, we reject no data, the arithmetic mean ($\pm$S.D.) permeability coefficients become 3-fold greater at $9.61 \pm 15.5 \times 10^{-5}$ cm/h ($n = 644$) for 5-FU and $11.7 \pm 15.1 \times 10^{-3}$ cm/h ($n = 221$) for ES, values evidently increased through including data for highly permeable skin. The Kolmogorov-Smirnov values and the Davis test clearly show that our accepted data are log-normally distributed. Literature reports of this phenomenon are scarce (Kastings et al., 1987; Williams, 1990); Kastings et al. reported that for five replicate penetration measurements the flux data for 35 compounds were found to be log-normally distributed. However, although this trend was mentioned, the authors did not pursue this finding. More recently, variability of flux data has been suggested to be dependent on the permeant used (Liu et al., 1991). The authors report that for neutral molecules the flux data are symmetrically distributed whereas ionic molecules show an asymmetrically positively skewed distribution. For log-normally distributed data, the geometric mean is the appropriate average value (Langley, 1979) although median values provide a close approximation (Kastings et al., 1987). Considering all our experimental data, arithmetic means tend to be higher than geometric means (Table 1) due to the nature of the skewed distribution. Additionally, the dispersion of the experimental data (expressed in terms of standard deviation) is greater when calculated arithmetically (161 and 138% for 5-FU and ES, respectively) than when calculated geometrically (113% for 5-FU, 75% for ES).

The implications of our results, if they apply to drug permeation in general, are clear; most statistical tests employed in the past to assess significance in a wide variety of skin protocols have tacitly assumed a normal distribution of data, probably because only 3–6 replicate measurements are determined. In order to employ, for example, the *t*-test to assess significance of penetration enhancer activities, Gaussian normality of drug permeability coefficients should ideally be proven for either the raw data or the data following a logarithmic transformation. When a log-normal distribution is indicated, then the arithmetic mean and standard deviation of the transformed data (or geometric values from the untransformed data) are required for a valid *t*-test. If insufficient data are acquired to prove a Gaussian distribution (of either raw or transformed data) then nonparametric tests such as Wilcoxon's signed ranks test or the Mann-Whitney U-test are more appropriate.

The practical implications arising from our data analysis are less clear. For some research protocols, investigators express experimental results as ratios of effects after treatment to those before, as in the penetration enhancing experiments mentioned previously. In such situations the inherent variability of the skin is, to some extent, corrected for, although the monitored response may itself be non-normally distributed. Where direct measurements of skin parameters are quoted, statistical use of arithmetic values will tend to underestimate significant differences when geometric values may indicate significance. Alterations to statistical decisions may only be evident where effects are marginally different, and in most situations use of arithmetic values will probably provide closely approximate decisions to those obtained if geometric values had been employed.

In conclusion, our data show that the permeability coefficients of 5-fluorouracil and oestradiol through human skin in vitro do not follow a normal distribution. The results clearly show a log-normal distribution and the appropriate average value to quote for such a distribution is the geometric mean. We appreciate that in the preparation of human epidermal membranes, tissue damage may be introduced. Indeed, the data distributions we have reported may be an artefact caused by performing permeation studies in vitro, and in vivo drug diffusion may follow a Gaussian dispersion; we would welcome comments from

other workers in skin research who may have similar extensive raw data banks available for re-analysis. However, for the large number of researchers who study percutaneous absorption in vitro (on either human or animal tissue) the potential existence of non-Gaussian distributions should be considered. The data we have presented relate to a function of human skin in vitro, not modifications caused by enhancers, an effect which will be addressed in a future communication.

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