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Towards a correlation between drug properties and in vitro transdermal flux variability

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Abstract

Over recent years, there has been growing evidence that the permeability coefficient variability describing any specific transdermal drug delivery system is not always normally distributed. However, since different researchers have used different test compounds, methodologies and skin types, it has been difficult to identify any general correlation between drug properties and flux variability. The aim of the present study was to investigate whether there was a relationship between these two variables. To this end, six different compounds (sucrose, adenosine, aldosterone, corticosterone, oestradiol and testosterone) exhibiting a range of partition coefficients but relatively similar molecular weights were screened by taking multiple replicate measurements of their permeation profiles as they penetrated across porcine skin in vitro. It was found that for relatively hydrophilic solutes ($\log P_{o/w} \leq \sim 2.5$), physicochemical properties that facilitated slow transdermal flux were associated with more positively skewed permeability coefficient distributions while rapid flux was associated with more symmetric distributions. However, no correlation could be found between molecular properties and the extent of statistical fit to either the normal or log-normal distribution.

Keywords: Transdermal; Permeability coefficient; Variability; Normal distribution; Log-normal distribution

1. Introduction

Over recent years, there has been mounting evidence that the permeability coefficient (K_p) values describing transdermal drug flux are not always distributed in a Gaussian-normal manner. As examples, it was reported that the variability of in vitro flux through dermatomed human skin was dependent upon the nature of the test penetrant (Liu et al., 1993). The flux data was symmetrically distributed for three neutral molecules but was highly positively skewed for two ionic drugs. Furthermore, analysis of flux data relating to in vitro tritiated water transport through human epidermis again provided evidence for non-normal distributions (Roper et al., 2000; Fasano et al., 2002). Other researchers (Williams et al., 1992; Cornwell and Barry, 1995) used fluorouracil as a model hydrophilic solute and oestradiol as a model lipophilic solute, and analysed both drugs' in vitro permeation through human epidermal membranes. Statistical evaluations again indicated positive skewing of the data. The K_p values for both compounds could be more closely fitted to log-normal frequency distributions than to normal frequency distributions. Similar findings have been reported in vivo. Each of the K_p databases describing the penetration of 10 non-steroidal anti-inflammatory drugs across human skin were positively skewed, approximating well with a log-normal distribution but not a normal distribution (Wenkers and Lippold, 1999).

Such non-normal K_p variability has far-reaching implications with regards to the statistical analysis of percutaneous drug penetration data. For example, use of the *t*-test to determine statistical significance assumes that the population exhibits Gaussian-normality. If this is not the case for K_p values then, strictly speaking, alternative non-parametric tests such as Wilcoxan's signed rank test or the Mann–Whitney *U*-test should be employed. Another possibility is to establish that the data exhibits Gaussian-normality following an appropriate arithmetic

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Table 1
Physicochemical properties of the selected test penetrants

Penetrant	log $P_{o/w}$ [a]	Molecular weight (Da)
Sucrose	−3.70	342
Adenosine	−1.05	267
Aldosterone	1.08	360
Corticosterone	1.94	346
Oestradiol	2.29[b]	272
Testosterone	3.32	288

[a] Values derived from published experimental data (Williams et al., 1992; Hansch et al., 1995).
[b] log $P_{o/w}$ values for oestradiol vary widely in the literature. We have used a value of 2.29 as described in Williams et al. (1992), Megrab et al. (1995), Barry (2002), Essa et al. (2002) and El Maghraby et al. (2005).

transformation. For instance, if the K_p database fits a log-normal distribution, the mean and standard deviation of the logged K_p data may be used for a valid *t*-test (Cornwell and Barry, 1995; Limpert et al., 2001).

One limitation of the transport studies described above is that due to the multiciplicity of drug types, membranes, methodologies and statistical tests used, it is difficult to discern any holistic trends relating molecular properties and permeation profile variability. The aim of the current study was to shed light on this issue by investigating whether there was a relationship between a drug's octanol–water partition coefficient and the resulting K_p data variability. A further objective was to investigate lag time distributions as this issue has attracted negligible research to date. To this end, six different solutes of comparable molecular weight but exhibiting different log octanol–water partition coefficient (log $P_{o/w}$) values were selected. This choice was made as it is already known that transdermal permeability is most influenced by partition coefficient and molecular weight (Potts and Guy, 1992; Vecchia and Bunge, 2003). Table 1 lists our candidate drugs – testosterone, oestradiol, corticosterone, aldosterone, adenosine and sucrose – with their corresponding molecular weights and log $P_{o/w}$ values (Hansch et al., 1995). The drugs were screened by taking multiple replicate measurements of their flux rates and lag times as they penetrated across porcine skin in vitro. Each penetrant's resultant K_p distribution was evaluated for normality by applying a Kolmogorov–Smirnov (KS) test while its asymmetry was quantified by applying a skewness test. KS tests were also employed to evaluate the normality of each drug's lag time distribution.

2. Materials and methods

2.1. Chemicals

All the radiolabeled penetrants *i.e.* [U-^{14}C]-sucrose (200 μCi/ml), [2-^{3}H]-adenosine (1 mCi/ml), [1,2-^{3}H]-aldosterone (1 mCi/ml), [1,2,6,7-^{3}H]-corticosterone (1 mCi/ml), [2,4,6,7-^{3}H]-oestradiol (1 mCi/ml) and [1,2,6,7-^{3}H]-testosterone (1 mCi/ml) were purchased from Amersham Biosciences (Amersham, UK). Most of the 'cold' penetrants *i.e.* adenosine, aldosterone, corticosterone, oestradiol, testosterone were purchased from Sigma–Aldrich (Poole, UK) as were phosphate buffer saline (PBS) tablets (pH 7.4) and absolute ethanol. 'Cold' sucrose was obtained from Amersham Biosciences (Amersham, UK). Optiphase HiSafe 3 scintillation fluid and scintillation vials were purchased from Fisher Scientific (Loughborough, UK) and Packard Instrument Co. (Meriden, CT), respectively. Deionized water was used throughout.

2.2. Preparation of skin samples

Porcine ears (Landrace species) were obtained immediately after slaughter from a local abattoir and cleaned under cold running water. These were sectioned by scalpel to yield whole skin samples, of area ∼8 cm^2 and depth ∼600–900 μm. Skin sections with no visible imperfections such as scratches or abrasions were then wrapped in aluminium foil and subsequently stored in a frozen state (−80 °C) for a maximum of 2 months before use. Prior to their use in the transport studies, the skins were allowed to thaw at room temperature and cut into smaller samples suitable for mounting on the diffusion cells.

2.3. Transport studies

Each skin sample was mounted in a static Franz diffusion cell (PermeGear, Bethlehem, PA), exhibiting a diffusional area of 0.64 cm^2 and a receptor compartment volume of 5.3 ml. In all the experiments, the receptor solution consisted of 10% (v/v) ethanol in PBS (pH 7.4) that had been degassed by sonication for 15 min (Camlab Transsonic T310, Cambridge, UK). This receptor phase was stirred at 600 rpm and maintained at 37 ± 0.5 °C by a thermostatic water pump (Haake DC10, Karlsruhe, Germany) which circulated water through each chamber jacket.

The skin samples were initially left in the Franz cells to hydrate for 1 h. Subsequently, 0.5 ml of donor solution, composed of the test solute in a 20:80% (v/v) mixture of ethanol:PBS (pH 7.4), was deposited onto each skin surface. Test solute concentrations were 1% (w/v) for sucrose, 0.1% (w/v) for adenosine and 0.01% (w/v) for the other four drugs. These concentrations were partially chosen to somewhat reflect maximal drug solubilities in the donor vehicle, which were 619 mg/ml for sucrose, 7.3 mg/ml for adenosine, 1.7 mg/ml for aldosterone, 1.3 mg/ml for corticosterone, 0.3 mg/ml for oestradiol and 0.5 mg/ml for testosterone (unpublished data). In all cases, sufficient radiolabeled compound was added and mixed with the 'cold' compound, so as to yield a donor solution activity of 1 μCi/ml. The amounts of drug in the donor compartment were such that an effective 'infinite dose' system existed. The donor compartments were always occluded with taught sheets of Parafilm® in order to prevent evaporative loss of the vehicle.

At selected time points (0, 1, 2, 3, 4, 5, 6, 20, 22, 24, 26 and 28 h) a 100 μl aliquot of solution was withdrawn from each receiver compartment and replaced with the same volume of blank receiver solution. Each 100 μl withdrawn aliquot was vortexed with 3 ml of scintillation fluid and permeant amounts in the withdrawn solutions were determined by liquid scintillation counting (Packard, TriCarb™ 1600TR). Each permeation study consisted of 63 replicates.

2.4. Data analysis

The permeant concentration values, derived from the scintillation counting, were corrected for progressive dilution using the equation:

$$M_t(n) = V_r C_n + V_s \sum C_m \quad (1)$$

where $M_t(n)$ is the current cumulative mass of drug transport across the skin at time t, C_n represents the current concentration in the receiver medium and $\sum C_m$ denotes the summed total of the previous measured concentrations [$m = 1$ to $(n-1)$]; V_r is the volume of the receiver medium and V_s corresponds to the volume of the sample removed for analysis.

Linear regression analysis was used to determine the gradient of the steady state segment of each permeation experiment. Each individual steady state flux value was divided by the solute concentration in the donor solution. This yielded a permeability coefficient (K_p) value for each individual replicate. All the replicate K_p values for each study were pooled together without the omission of any outliers. We first assessed how close each distribution fitted a Gaussian-normal pattern by applying the KS test (Miller and Miller, 1986). Notably, since the true mean and standard deviation of each entire K_p population remain unknown, we could not use the pure form of the KS test. Therefore, we applied a modified version of the test known as "Dallal and Wilkinson's approximation to Lilliefors' method" (Lilliefors, 1967; Dallal and Wilkinson, 1986). This version calculates, from the inputted sample values, estimates of the population mean and population standard deviation. Differences were deemed statistically significant when $P < 0.05$. IBM-compatible software, specifically Prism® version 4.03 (GraphPad Software, San Diego, CA), was used to undertake the KS tests. When the calculated P-values were greater than 0.10 or below 0.001, the software indicated this but did not yield the precise numerical value.

Each K_p distribution profile was also tested for skewness, which was calculated according to the following equation:

$$\text{skewness} = \frac{n}{(n-1)(n-2)} \sum \left[\frac{x_i - \bar{x}}{s} \right]^3 \quad (2)$$

where n is the sample size, x_i represents the ith value, $\bar{x}$ denotes the sample mean and s is the representative of the sample standard deviation. The IBM-compatible software package, Minitab™ (Minitab Inc., State College, PA), was utilized to run the skewness tests.

Linear regression analysis was also employed in order to isolate the lag time value for each experimental replicate. For each drug, all the replicate lag time values were pooled together without the omission of outliers. Each distribution was tested for normality using the modified KS test described above.

3. Results

3.1. Penetration–time data

Fig. 1 presents the pooled penetration–time plots for all six investigated test compounds. It is noteworthy that each drug

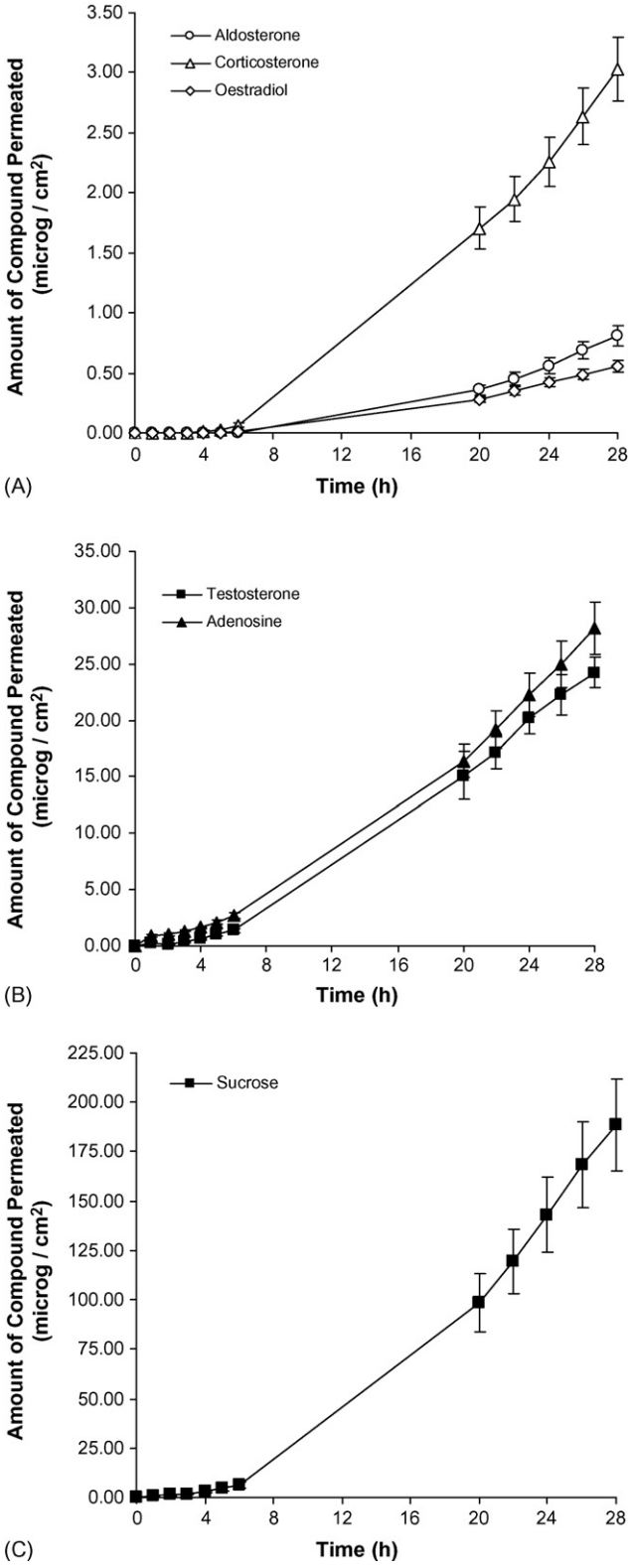

Fig. 1. Penetration of candidate drugs through full thickness porcine skin as a function of time. Error bars represent standard error of the mean values, $n = 63$. (A) Aldosterone, corticosterone, oestradiol profiles. (B) Testosterone and adenosine profiles. (C) Sucrose profile.

penetration–time plot represents an arithmetic composite of 63 replicate plots with no omitted outliers. Importantly, the pooling together all 63 replicate plots can result in a somewhat shape-distorted arithmetic mean plot. This is because the inclusion of several highly permeable outliers will influence the arithmetic mean plot more greatly at high time points than at low time points. This can yield a false optical perception that steady state has not definitively been attained (as for example is particularly noticeable in the corticosterone, aldosterone and oestradiol profiles over 20–28 h) and/or that the lag time is longer than it really is. We could remedy this profile distortion effect by plotting the geometric mean profile of the 63 replicate plots, or alternatively by omitting the highly permeable outliers. However, these transport plots are inclusive of all data and we do not omit outliers as we are trying analyse variability rather than omit it.

To re-emphasise, it must be remembered that lag time and steady state selection was performed individually on each replicate run. Indeed, approximately 90% of all selected steady state segments exhibited excellent linearity ($0.99 \leq r^2 \leq 1.00$) while the remainder displayed reasonable linear correlations ($0.91 \leq r^2 \leq 0.99$).

Calculations showed that sink conditions were maintained throughout the experimental duration in all six studies. Thus, the experimental conditions allowed free diffusion for all the candidate drugs.

3.2. Mean K_p values

Table 2 lists the mean K_p values for each of the candidate permeants. For testosterone, the measured mean K_p value was 11.33×10^{-3} cm/h. This is reasonably close to a value of 7.9×10^{-3} cm/h obtained by others (Qvist et al., 2000) who also tracked in vitro testosterone permeation through full-thickness pig skin, albeit using slightly different conditions. For oestradiol, we could not find any literature data relating to this drug's permeation through whole porcine skin. However, our measured value of 4.24×10^{-3} cm/h was quite close to a value of 3.89×10^{-3} cm/h documented for in vitro oestradiol penetration through full-thickness human skin (Galey et al., 1976). It is noteworthy that for the four investigated steroids, the mean K_p values increased with increasing lipophilicity in a manner consistent with previous (El Tayer et al., 1991; Knutson et al., 1993; Johnson et al., 1995) and contemporary (Magnusson et al., 2006) reports.

3.3. K_p distributions: results of the Gaussian-normality tests

Fig. 2 shows the K_p frequency distribution data for all six investigated test drugs. Subjective visual observation suggests that all the penetrants except oestradiol exhibit asymmetric K_p distribution profiles, characterised by considerable tailing towards higher permeability coefficient values. The oestradiol profile appears more symmetrical with perhaps only minor positive tailing.

Modified KS tests were undertaken in order to quantitatively assess the extent to which each of these K_p distributions fits a Gaussian-normal distribution. Table 2 presents the KS results. The key parameter is the KS distance, whose value would equal zero if the permeant's K_p values were distributed in a perfect Gaussian-normal configuration. Larger values of the KS distance correspond to larger deviations from an ideal Gaussian distribution. By combining the KS distance and sample size, it is possible to calculate a *P*-value that represents the chance that the sample was derived from a normally distributed parent population. As can be seen from Table 2, the results indicated that the distribution of both oestradiol and testosterone K_p values closely fitted a Gaussian-normal pattern. In other words, the visually apparent deviations from normality for these two penetrants can be explained by random fluctuations associated with the relatively small size ($n = 63$) of our sample relative to the size of the population ($n = \infty$). In contrast, the K_p distributions of sucrose, cimetidine, aldosterone and corticosterone did not pass the normality test.

Since many reports have indicated that transdermal K_p values tend to be distributed in a log-normal manner, this possibility was also assessed by logging all the individual K_p values and running the KS test on the transformed data. Table 3 presents the statistical results. It can be seen that while the oestradiol data did not correlate well with a log-normal tendency the other five penetrants could be fitted to such a profile.

3.4. K_p distributions: results of the skewness tests

The skewness statistic quantifies the degree of symmetry of a distribution around its mean. This will range from zero for a perfectly symmetrical distribution to progressively larger values with increasing positive skewness *i.e.* more heavier tailing on the right hand side of the distribution. Fig. 3 presents a graph relating K_p distribution skewness as a function of mean K_p for

Table 2
Statistical analysis of permeability coefficient data

Penetrant	$K_p \times 10^{-3}$, mean ± S.E.M. ($n = 63$)	KS distance	*P*-value	Passed normality test? (* = 0.05)
Sucrose	1.206 ± 0.132	0.221	<0.001	No
Adenosine	1.584 ± 0.114	0.157	<0.001	No
Aldosterone	0.586 ± 0.054	0.160	<0.001	No
Corticosterone	1.808 ± 0.139	0.149	0.001	No
Oestradiol	4.248 ± 0.310	0.071	>0.100	Yes
Testosterone	11.330 ± 0.906	0.119	>0.100	Yes

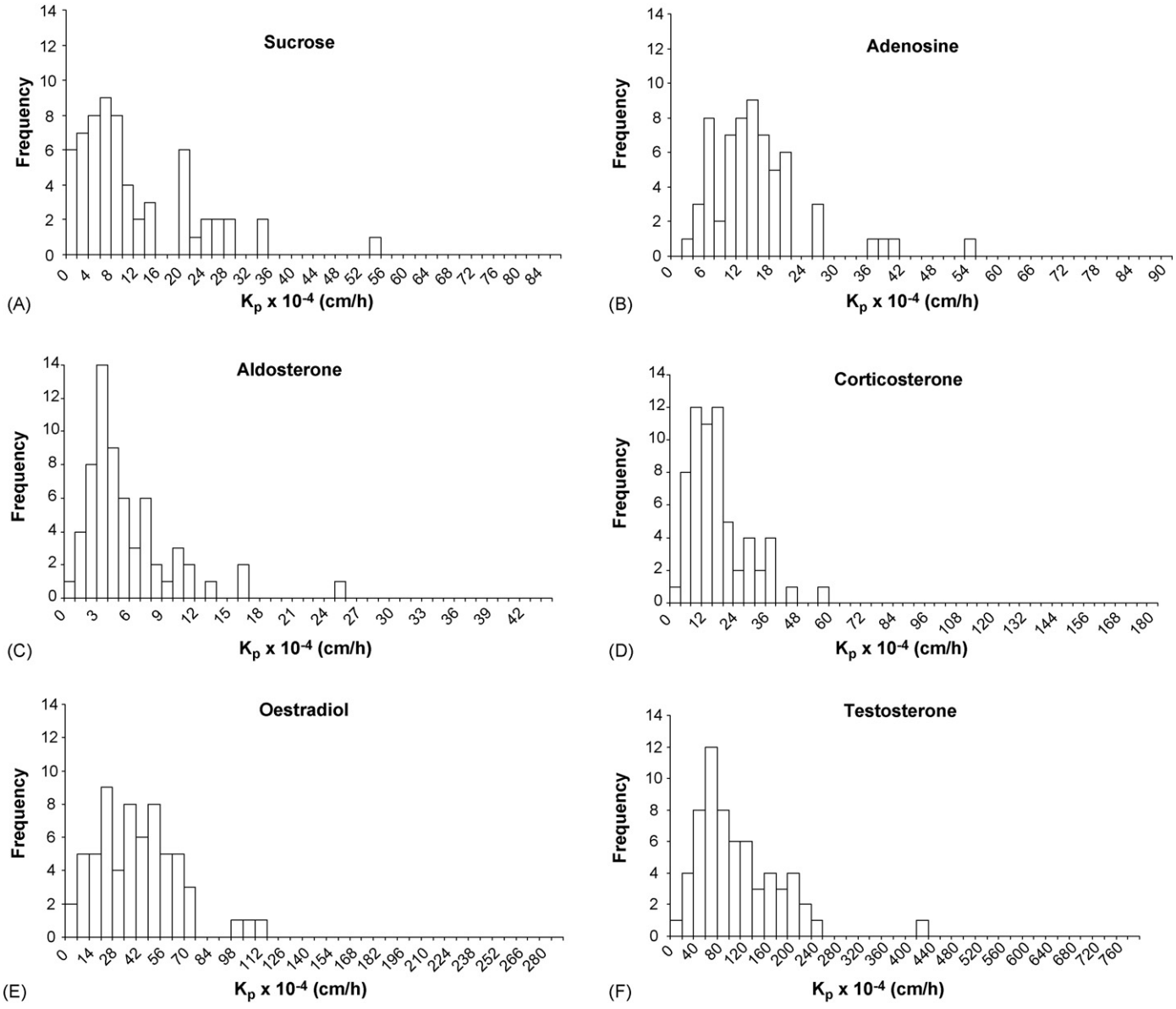

Fig. 2. Permeability coefficient frequency distributions for all test compounds.

all of the candidate penetrants. It can be seen that apart from testosterone, there seems to be a correlation between the rate of solute permeation and the skewness of the distribution. More rapidly permeating solutes tend to exhibit more symmetric distributions while more slowly penetrating drugs tend to exhibit greater positive tailing.

3.5. Lag time distributions

Fig. 4 presents the lag time frequency distribution histograms for all of the test compounds while Table 4 compiles the results of KS analysis on this data. It is noteworthy that that the lag time variabilities for sucrose, adenosine, aldosterone, cortiscosterone

Table 3
Statistical analysis of log-transformed permeability coefficient data

Penetrant	log K_p, mean ± S.E.M. ($n = 63$)	KS distance	P-value	Passed normality test? (* = 0.05)
Sucrose	−3.088 ± 0.053	0.077	>0.100	Yes
Adenosine	−2.859 ± 0.029	0.086	>0.100	Yes
Aldosterone	−3.324 ± 0.036	0.061	>0.100	Yes
Corticosterone	−2.819 ± 0.034	0.052	>0.100	Yes
Oestradiol	−2.459 ± 0.038	0.121	0.022	No
Testosterone	−2.030 ± 0.036	0.062	>0.100	Yes

Table 4
Statistical analysis of the lag time data

Penetrant	Lag time (h), mean ± S.E.M. ($n = 63$)	KS distance	P-value	Passed normality test? (* = 0.05)
Sucrose	13.27 ± 0.36	0.091	>0.100	Yes
Adenosine	10.45 ± 0.37	0.097	>0.100	Yes
Aldosterone	14.73 ± 0.32	0.080	>0.100	Yes
Corticosterone	11.78 ± 0.36	0.096	>0.100	Yes
Oestradiol	12.50 ± 0.29	0.056	>0.100	Yes
Testosterone	6.73 ± 0.46	0.284	<0.001	No

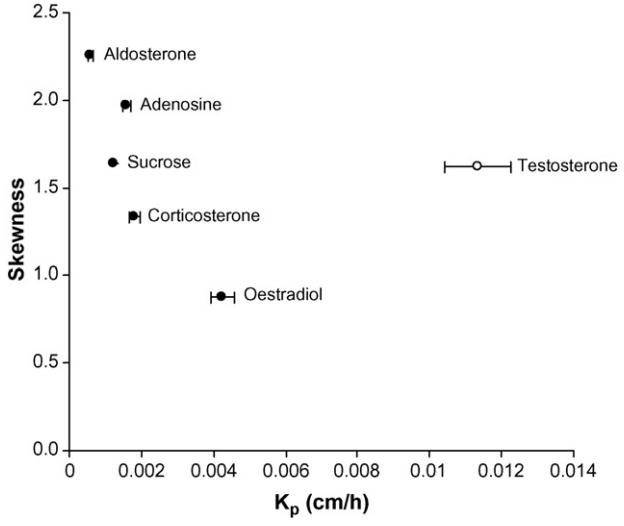

Fig. 3. The relationship between K_p distribution skewness and mean K_p for each of the test solutes. Error bars represent standard error of the mean values, $n = 63$. (●) Penetration limited by the stratum corneum barrier. (◯) Penetration limited by the hydrophilic layers.

and oestradiol could be easily fitted to normal distributions. Furthermore, these four solutes had similar lag times, with mean values ranging between approximately 10.5 and 14.7 h. In contrast, the lag time variability for testosterone could not be fitted to a normal distribution. This effect was quite marked and subjective visual observation of the relevant histogram suggests this penetrant exhibited a pronounced bimodal distribution profile. Specifically, one set of skin sections displayed a mean lag time of ∼5 h while another smaller group of samples demonstrated a mean lag time of ∼13 h. Lag time assessments should generally be treated with some caution as determinations are highly subjective—small differences in individual identification of the steady state segment often produce large differences in lag time values. Nevertheless, since all our data was analysed by the same researcher, we feel that the results can be reasonably compared.

4. Discussion

The first issue to consider was whether the K_p variabilities of the test solutes were normally distributed. Our results showed that only the K_p databases of the two most lipophilic compounds correlated well with a Gaussian-normal pattern while the K_p databases of the other four drugs could not be fitted to such a pattern. Despite this initial finding, plotting the KS distance as a function of $\log P_{o/w}$ for all six solutes did not yield a clear, convincing correlation between the two parameters. It is possible that a good correlation would have emerged if the sample size was greater than six.

Another question to consider was whether K_p distributions of the compounds were log-normally distributed. This issue arises because although the normal distribution is more popular, there is evidence that the log-normal distribution is more relevant for describing the variability of membrane transport phenomena. This paradigm follows the fact that the arithmetic relationship between individual physical transport steps is multiplicative in nature rather than additive (Limpert et al., 2001). Within the context of percutaneous drug delivery, log-normal K_p distributions have been reported to describe; in vitro 5-fluorouracil and oestradiol flux through human epidermis (Williams et al., 1992), in vitro tritiated water flux through rat epidermis (Fasano et al., 2002) as well as the percutaneous absorption of 10 different non-steroidal anti-inflammatory drugs in human volunteers (Wenkers and Lippold, 1999). Furthermore, in a previous paper we determined that the variability of caffeine penetration through porcine skin was log-normal in nature (Khan et al., 2005). In the present study, it was found that the K_p databases of five of the test compounds correlated well with a log-normal distribution and that only the oestradiol K_p database could not be fitted to this distribution. It is noteworthy that the testosterone K_p data could be fitted to both a log-normal and normal distribution but that this is actually statistically permissible (Limpert et al., 2001). Unfortunately, plotting KS distance as a function of $\log P_{o/w}$ for all six compounds did not yield a convincing correlation between the two parameters. In addition, the authors of this paper have attempted to fit various mathematical models to this data but no meaningful relationship could be found between any molecular or kinetic parameters and the deviation from log-normal variability.

It is crucial to realise that normality and by extension log-normality are composite characteristics. A distribution will only be normal if it exhibits sufficient symmetry, a suitable degree of kurtosis ("peakedness") as well as a unimodal tendency. We performed a skewness analysis in an attempt to deconvolute the symmetry parameter from the more complex normality test. Interestingly, the results indicated that for the five more hydrophilic solutes, there was a trend relating mean flux-to-flux data symmetry. More slowly penetrating compounds tended to exhibit more positively skewed K_p distributions while more rapidly penetrating drugs exhibited more symmetric K_p distributions. In order to explain this effect, it is necessary to consider

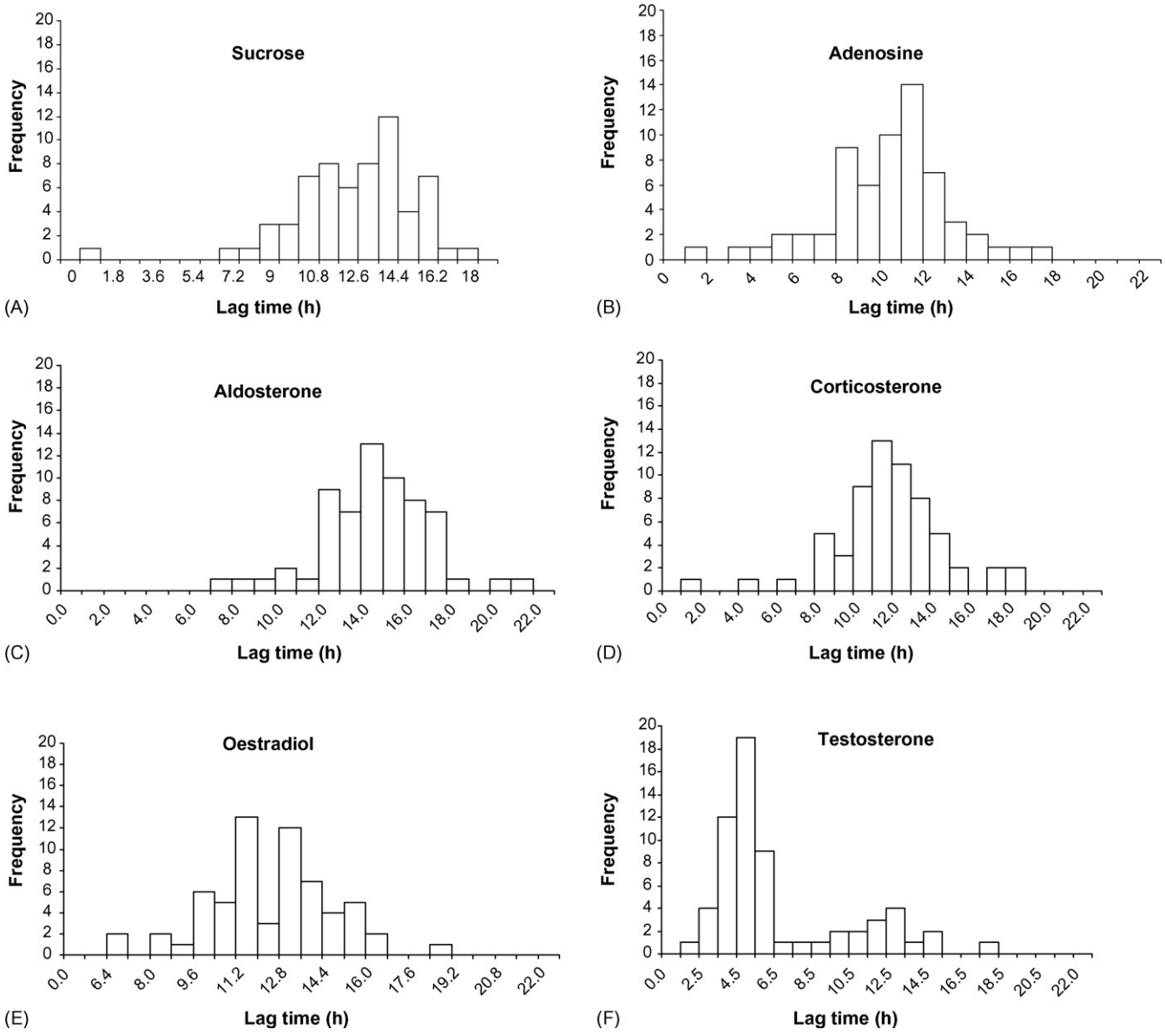

Fig. 4. Lag time frequency distributions for all test compounds.

that the positive tails of each distribution are associated with individual experiments performed on more permeable skin samples. Such greater than average permeability is probably due to stratum corneum imperfections such as abrasions, hair follicle openings or other areas exhibiting reduced barrier function. Crucially, it is likely that such defects will have a larger relative impact for molecules that slowly penetrate through the continuous stratum corneum rather than for molecules that rapidly penetrate through this layer. However, testosterone appeared as an outlier on the plot of skewness versus mean K_p It should be remembered that testosterone is sufficiently lipophilic that the rate-limiting step in its penetration is likely to be transport from the stratum corneum to the underlying hydrophilic layers (Magnusson et al., 2006). Hence, this compound is unaffected by the paradigm described above.

Finally, it was found that testosterone exhibited bimodally distributed lag times while the five drugs exhibited normally distributed lag times. This suggests that for testosterone, the initial non-steady state phase of absorption involves two different pathways with individual skin samples tending to offer only one of these pathways. In contrast, the other solutes may penetrate via only one major pathway during this initial phase. Since the lag time is dependent upon both the pathlength as well as the solute's diffusion coefficient, new experiments would be required in order to separate out these two variables so that the full mechanistic implications can be discerned.

In conclusion, our data provides evidence that for relatively hydrophilic drugs ($\log P_{o/w} \leq \sim 2.5$), there is a correlation between mean K_p and the symmetry of the K_p data. In other words, compounds that have physicochemical properties that facilitate rapid transdermal flux will tend to have more symmetrical K_p distributions while slower flux is associated with increasing positive skewness. However, no meaningful relationship could be found between molecular properties and the extent

of fit to either the normal or log-normal distribution. We are aware of the fact that four out of our six test compounds were steroids. Therefore, further work involving a larger number of solutes of different chemical classes, is clearly warranted in order to obtain a more comprehensive understanding of this field.

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