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In vitro percutaneous absorption evaluation of phenobarbital through hairless mouse, adult and premature human skin

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Summary

In vitro phenobarbital flux through newborn (preterm and full-term) infant, hairless mouse and adult human skin was determined using both Franz cells and flow-through diffusion cells. The phenobarbital flux value through preterm infant skin was significantly higher than that obtained through full-term infant skin which, in turn, was close to that measured for adult human skin. No significant difference was observed between phenobarbital flux values through preterm infant skin and hairless mouse skin using flow-through diffusion cells: this result suggests that hairless mouse skin can be successfully used as a model to study in vitro percutaneous absorption of phenobarbital through preterm infant skin. Phenobarbital flux through preterm infant skin was affected by the gestational age since flux decreased as the gestational age increased and from 37 weeks gestation onward (full-term infants) flux values were similar to those determined for adult human skin. Since by using the flux value from the in vitro experiments on preterm infant skin a steady state plasma concentration close to the therapeutic level can be predicted, phenobarbital transdermal delivery in preterm infants could be regarded as feasible.

Introduction

Neonatal drug therapy often presents a number of problems such as erratic and unpredictable absorption after oral drug administration (Jones and Baillie, 1979) and difficulties in intravenous administration with vein location, fragility and because drugs are given together with fluid whose rate of administration cannot be changed without altering the amount of drug given.

Recently, transdermal drug delivery has been regarded as a particularly suitable route for drug administration in newborn infant therapy because of: (1) greater permeability of the preterm infant's skin; (2) very high surface area to weight ratio; (3) relatively low volume of distribution, metabolism and excretion; (4) very small dose required to achieve therapeutic effects (Evans and Rutter, 1989). Furthermore, the transdermal route is not

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invasive and does not require additional fluids for administration.

Therefore, drugs which cannot be administered transdermally in adults due to their physicochemical and/or pharmacokinetic properties (e.g., low skin premeability, high therapeutic dosage) can be regarded as candidates for transdermal administration in preterm infant therapy.

The most commonly used drugs for neonatal (preterm and full-term) infant therapy are antibiotics, theophylline, frusemide, indomethacin and anticonvulsants. Among these drugs, only theophylline has been studied for transdermal delivery in preterm infants. Evans et al. (1985) have reported that theophylline sodium glycinate can be administered percutaneously to preterm infants and theophylline therapeutic levels can be achieved within a few hours and maintained for several days.

Phenobarbital is the drug of choice for generalized tonic-clonic seizures in newborn infants (Lockman et al., 1979). No information on its flux values through newborn (full-term and preterm) infant skin has been reported, thus it is not possible to assess whether phenobarbital can be delivered transdermally in newborn infant therapy.

The aim of this investigation was to determine phenobarbital in vitro flux through newborn (preterm and full-term) infant skin so as to evaluate the feasibility of phenobarbital transdermal administration in newborn infants. Since it is difficult to obtain newborn infant skin samples for in vitro studies, we included adult human and hairless mouse skin in our investigation so as to establish which of these two skin types could provide phenobarbital percutaneous flux values comparable to those determined using newborn infant skin.

Materials and Methods

Materials

[2-^{14}C]Phenobarbital (specific activity 29 mCi/mmol) was purchased from Amersham (U.K.). Phenobarbital was bought from Sigma (St. Louis, MO).

All other chemicals and solvents were of analytical grade.

Preparation of skin membranes

Samples of whole adult human skin (mean age 45 ± 15 years) were obtained from breast reduction operations. Subcutaneous fat was carefully trimmed and the skin was immersed in distilled water at 60 ± 1°C for 2 min (Kligman and Christophers, 1963), after which the stratum corneum and epidermis (SCE) were peeled off. Samples of neonatal skin (preterm 29–35 weeks of age and full-term 37–40 weeks of age, male and female) were removed from the midline region of the chest within 48 h of death. The stratum corneum and the epidermis were separated from the dermis using the same procedure described above but immersing the preterm infant skin samples in distilled water at 60 ± 1°C for 1 min. The SCE samples were dried at room temperature in a desiccator maintained at approx. 25% RH. The dried samples were wrapped in aluminium foil and stored at 4 ± 1°C. Samples of dried SCE were rehydrated by immersion in distilled water at room temperature for 1 h before being mounted in diffusion cells.

Female hairless mice, 6–9 weeks old, weighing 24–30 g, were purchased from Temple University (Philadelphia, PA). The mice were killed by cervical dislocation; the skin from the dorsal region was removed and the subcutaneous fat and other extraneous tissues trimmed. The whole skin was immersed in 0.1 M phosphate buffered saline containing 20 mM EDTA and incubated for 3–4 h at 37 ± 1°C. After incubation, the stratum corneum and the underlying epidermis were peeled off in accordance with the procedure of Koch et al. (1989). SCE samples were immediately used in diffusion experiments.

In vitro skin permeation experiments

Skin permeation of phenobarbital was measured using both Franz cells and flow-through diffusion cells. Franz cells were similar to those described by Franz (1975). The receiving chamber had a volume of 6.7 ± 0.3 ml and was filled with saline. The receptor phase was stirred and kept

at 35 ± 1°C during the experiment. The surface area for absorption was 2.0 cm^2.

The flow-through diffusion system was supplied by LGA (Berkeley, CA). The receiving chamber had a volume of 3.0 ml and the area available for diffusion between compartments was 1.0 cm^2. The temperature of the receiving chamber was kept at 35 ± 1°C and receptor phase was stirred during the experiments. Normal saline was pumped into the receiving chamber at a flow rate of 18 ml/h.

Phenobarbital was dissolved in ethanol (2 mg/ml, 7.0 μCi) and 39 $\mu l/cm^2$ was placed on the stratum corneum surface. The solvent was allowed to evaporate and each experiment was run for 12 h. Using Franz cells, samples of the receiving solution (50 μl) were withdrawn at intervals and replaced with fresh solution. The samples were placed in 20 ml liquid scintillation coktail (Insta-Gel, Packard) and analyzed for [2-^{14}C]phenobarbital content with a Beckman LS 9800 series liquid scintillation counter (85–95% efficiency).

Using flow-through diffusion cells, the effluent from the receiving chamber was collected in test tubes using a fraction collector at 1-h intervals over 12 h. From each sample, two 1-ml aliquots were taken and [2-^{14}C]phenobarbital content was measured as described for Franz cells.

Calculations

The fluxes through the skin of phenobarbital were evaluated, using linear regression analysis, by plotting the cumulative amount of phenobarbital permeated against time and dividing the slopes of the steady state portion of the graphs by the area of the diffusion cells. Lag time was calculated from the x-intercept values of the regression lines. Each measurement was made in duplicate.

Statistical analysis of the data was performed using Student's t-test.

Results and Discussion

In vitro skin permeation experiments were performed using SCE membranes instead of full-thickness skin since, as reported by others (Scheuplein and Blank, 1973; Bronaugh and Stewart, 1984), the dermis in vitro can act as a significant artificial barrier to the absorption of lipophilic compounds. Phenobarbital flux was determined using both Franz cells and flow-through diffusion cells. The latter was chosen because it has been reported that it provides a more reliable assessment of the percutaneous penetration of drugs, since it mimics the effect of blood flow through the skin by taking up and carrying away the permeated drug (Bronaugh and Stewart, 1985).

TABLE 1

Phenobarbital fluxes and lag time values through hairless mouse, preterm and full-term infant and adult human skin determined using Franz cells and flow-through diffusion cells

Skin sample	n	Cell type	Flux (±S.D.) ($\mu g\ cm^{-2}\ h^{-1}$)	Lag time (h)
Preterm infant	4	FC [a]	0.50±0.26	1.5±0.8
	4	FTC [b]	0.41±0.13	0.5±0.2
Full-term infant	3	FC	0.10±0.03	1.8±0.8
	3	FTC	0.12±0.02	2.2±0.7
Hairless mouse	6	FC	1.73±0.74	1.1±0.6
	5	FTC	0.72±0.24	2.6±0.6
Human adult	3	FC	N.D. [c]	N.D. [c]
	3	FTC	0.10±0.02	2.7±1.2

[a] FC, Franz cells.
[b] FTC, flow-through diffusion cells.
[c] N.D., no phenobarbital flux was detectable.

In vitro flux of phenobarbital through preterm and full-term infant, adult human and hairless mouse skin is reported in Table 1. Statistical analysis results obtained after application of Student's t-test to the flux values of phenobarbital through preterm infant skin vs flux values through full-term infant, adult human and hairless mouse skin are reported in Table 2.

The phenobarbital flux value through preterm infant skin was significantly higher ($p < 0.05$) than that obtained through full-term infant skin which, in turn, was close to that measured for adult human skin. These results agree well with the finding that mature newborn infants have an effective epidermal barrier whose permeability to drugs is similar to that of adult skin (Harpin and

TABLE 2

Comparison of phenobarbital fluxes through hairless mouse, preterm and full-term infant and adult human skin using Student's t-test

Comparison in terms of barrier membrane	Significance of difference	
	FC [a]	FTC [b]
Preterm vs full-term infant skin	$p < 0.05$	$p < 0.05$
Preterm infant skin vs hairless mouse skin	$p < 0.05$	$p > 0.05$
Preterm infant skin vs adult human skin	N.C. [c]	$p < 0.05$
Full-term infant skin vs hairless mouse skin	$p < 0.05$	$p < 0.05$
Full-term infant skin vs adult human skin	N.C. [c]	$p > 0.05$
Hairless mouse skin vs adult human skin	N.C. [c]	$p < 0.05$

[a] FC, Franz cells.
[b] FTC, flow-through diffusion cells.
[c] N.C., not calculated since no flux through adult human skin was detectable.

Rutter, 1983), while preterm infant skin permeability to drugs is higher (Evans and Rutter, 1986).

Since hairless mouse skin has been reported as more permeable than adult human skin to a great number of drugs (Wester and Noonan, 1980), we determined in vitro phenobarbital flux through hairless mouse SCE in order to assess whether flux values similar to those measured using preterm infant SCE could be obtained. As can be seen from Tables 1 and 2, no significant difference was observed between phenobarbital flux values through hairless mouse SCE and preterm infant SCE using flow-through diffusion cells. Using Franz cells, flux values through hairless mouse skin were significantly higher than those through preterm infant skin and those obtained using flow-through cells. Since the flux values through hairless mouse skin using Franz cells showed a high standard deviation we believe that the differences could not be regarded as really significant.

The results obtained suggest that hairless mouse SCE can be successfully used as a model to study in vitro percutaneous absorption of phenobarbital through preterm infant skin.

Other authors (Bailey and Briggs, 1983) have studied the in vivo percutaneous absorption of phenobarbital sodium salt through hairless mouse skin and found that it was absorbed and accumulated in the liver. Unfortunately, no comparison can be made with our results since we used phenobarbital instead of its sodium salt and it is well known that the percutaneous absorption of a molecule in its ionic form is different from that in the nonionic form.

As can be noted from Table 2, using Student's *t*-test for comparing flux values obtained with Franz cells and flow-through diffusion cells a significant difference was observed measuring phenobarbital flux through adult human skin: no flux was detectable using Franz cells while a flux, even if low (0.10 $\mu g\ cm^{-2}\ h^{-1}$), was observed using flow-through diffusion cells. For this last system, the flow rate of the receptor fluid was not found to influence phenobarbital flux values.

The detection level for phenobarbital flux was 0.08 $\mu g\ cm^{-2}\ h^{-1}$. The different data obtained for adult human skin using Franz cells and flow-through cells could be explained by taking into account the different receptor phase volume used for determining the amount of drug permeated: 50 μl in the case of Franz cells and 1 ml for flow-through cells. Since the receptor phase volume withdrawn from Franz cells was very small, probably no flux could be detected during the experiment period because the amount of phenobarbital permeated was under the detection limit. Nevertheless, in some cases, we noted small amounts of phenobarbital in the receptor phase of Franz cells during the last 3–4 h of the experiments but the data did not allow us to determine the flux values.

On comparison of the lag times obtained in our experiments (see Table 1), we observed that the percutaneous absorption of phenobarbital through full-term infant and human adult SCE showed the greatest lag time while those obtained from hairless mouse and preterm infant SCE were comparable. Statistically, however, the comparison of the lag time values for all four showed no significant difference since standard devia-

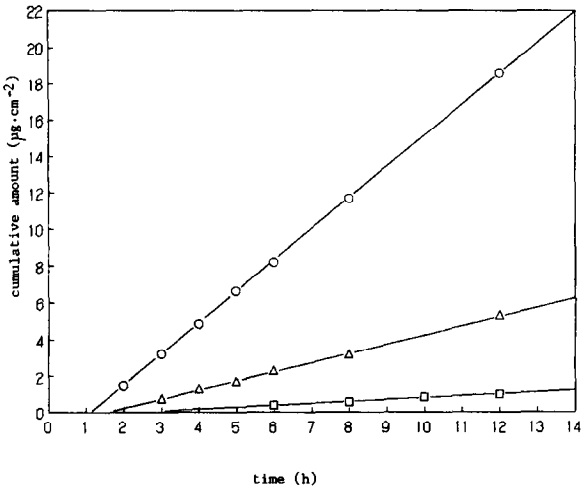

Fig. 1. Comparison of phenobarbital penetration through hairless mouse skin (○), preterm infant skin (△) and full-term infant skin (□), using Franz cells.

tions were high. Plotting the cumulative amount of phenobarbital permeated against time we obtained the plots reported in Figs 1 (Franz cells) and 2 (flow-through diffusion cells). As can be seen, pseudo-steady state conditions exist during the 12 h period of our experiments and data points from the linear portion of the graph were used to calculated the regression slope.

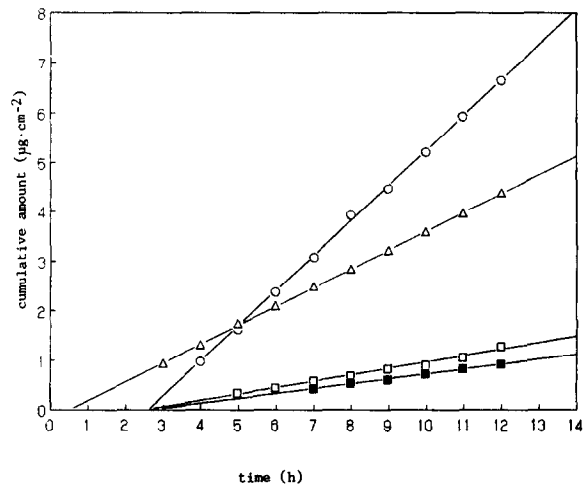

Fig. 2. Comparison of phenobarbital penetration through hairless mouse skin (○), preterm infant skin (△), full-term infant skin (□) and adult human skin (■) using flow-through diffusion cells.

TABLE 3

Phenobarbital fluxes and lag time values determined using flow-through diffusion cells for newborn infant (preterm and full-term) skin samples with different gestational and postnatal age

Gestational age (weeks)	Postnatal age (days)	Flux (μg cm^{-2} h^{-1})	Lag time (h)
29	3	0.53	0.5
30	4	0.47	0.3
33	3	0.39	0.6
35	2	0.24	0.7
37	5	0.11	2.3
38	2	0.11	2.9
40	5	0.14	1.5

$n = 1$.

Since many authors have observed that preterm infant skin permeability is strongly affected by the gestation and postnatal age (Nachman and Esterly, 1971; Harpin and Rutter, 1983; Barker et al., 1987) we report these parameters in Table 3 together with phenobarbital flux through newborn infant skin.

As can be seen in Fig. 3, our data agree well with those of previous authors, since flux through preterm infant skin decreased as the gestational age increased and from 37 weeks gestation onward (full-term infants) phenobarbital flux values were similar to those obtained for adult human skin. The difference in skin permeability between preterm and full-term infant skin has been explained on the basis of the different maturation of the epidermis. While the term infant shows a

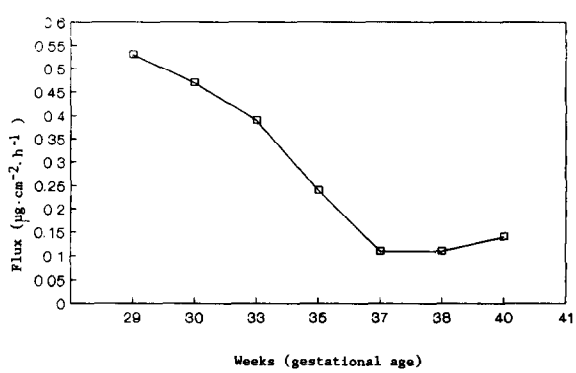

Fig. 3. Relationship between in vitro phenobarbital flux through newborn (preterm and full-term) infant skin and gestational age ($n = 1$).

well developed epidermis and a well-formed stratum corneum, resembling that of a child or adult, a preterm infant has a poorly developed epidermal barrier (Evans and Rutter, 1986). Preterm infant skin permeability is also affected by the postnatal age: as epidermis quickly matures after birth, skin permeability rapidly falls to term levels by about 3 weeks of age (Harpin and Rutter, 1983). West et al. (1986) reported that benzoic acid flux after topical application to preterm infants is closely related to both gestational and postnatal age. In our experiments it was not possible to establish a relationship between phenobarbital flux through the skin and postnatal age, since all our samples were obtained from newborn dead of almost the same age.

To assess the feasibility of phenobarbital transdermal adiministration in preterm infants, blood levels of phenobarbital following application of a transdermal formulation were predicted by using the flux value from the in vitro experiments on preterm infant skin (see Table 1). Steady-state plasma concentration (C_{SS}) can be calculated by means of Eqn 1 (Touitou et al., 1988):

$$C_{SS} = J \times A / V_d \times K_e \quad (1)$$

where J is the slope of the linear section of the cumulative amount permeated per unit area vs time plot, A denotes the area of application to the skin, V_d (l/kg) is the volume of distribution, and K_e (h) represents the elimination constant. V_d and K_e of phenobarbital in preterm infants are 0.54 l/kg and 0.6×10^{-2} h^{-1}, respectively (Goodman and Gilman, 1985). Thus, for a formulation delivering phenobarbital and having a flux of 0.41 $\mu g\ cm^{-2}\ h^{-1}$, a blood concentration of 3.16 $\mu g/ml$ is predicted for an area of application to the skin of 25 cm^2 for a 1 kg baby. This is only an approximate estimation since biotransformation of phenobarbital in its penetration through the preterm infant skin is not known.

Since the calculated value of C_{SS} for phenobarbital is close to the plasma therapeutic concentration in children reported in the literature (5–7 $\mu g/ml$) (Goodman and Gilman, 1985), phenobarbital transdermal delivery in preterm infants could be regarded as feasible. Applying Eqn 1 to calculate C_{SS} for full-term infants, assuming that V_d and K_e are the same, the C_{SS} value is not suitable for transdermal administration. Since phenobarbital shows a long half-life in preterm infants (115 h), a long time for the plasma concentration to achieve a steady-state could be expected. This problem should be taken into account in the design of a transdermal system for delivering phenobarbital in premature infants.

In conclusion, in vitro phenobarbital flux through preterm infant skin was higher than using human adult and full-term infant skin and was dependent on the gestational age. Since phenobarbital flux values through preterm infant and hairless mouse skin were comparable, the latter can be regarded as a good model for preterm infant skin with respect to the in vitro percutaneous penetration of phenobarbital. Since by using the phenobarbital flux value through preterm infant skin an approximate blood steady-state concentration close to the therapeutic level can be predicted, we believe that phenobarbital can be regarded as a candidate for transdermal administration in preterm infant therapy. Clinical in vivo studies are needed to validate the feasibility of phenobarbital transdermal delivery in preterm infants.

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