J. Pharm. Pharmacol. 1986, 38: 726–730
Received March 24, 1986

An approach to reduce the number of skin samples in testing the transdermal permeation of drugs

P. LANGGUTH*, H. SPAHN, E. MUTSCHLER AND K. HÜBNER†

Pharmakologisches Institut für Naturwissenschaftler der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, Gebäude 75 A D-6000 Frankfurt am Main, and †Senckenbergisches Zentrum der Pathologie Universitätsklinikum Frankfurt, Theodor-Stern-Kai 7, Gebäude 6, D-6000 Frankfurt am Main, FRG

Although glyceryl trinitrate (GT) is a drug that easily permeates through skin, the variations in its transepidermal fluxes were high. The arithmetic mean of the GT flux (n = 31 skin samples from different individuals) was 16·5 μg cm^{-2} h^{-1} with a standard deviation of 42%. The extreme values were 4·1 and 36·9 μg cm^{-2} h^{-1}, i.e. they differed by a factor of 9. Wide variations were also found for ephedrine, frusemide, caffeine, ethacrynic and benzoic acids and especially trospium chloride. All these fluxes were determined in an in-vitro permeation model at 32 °C using human epidermis. With the aim of standardizing epidermal preparations on their permeability, the extent to which the in-vitro GT fluxes through a human epidermal preparation correlate with those of other compounds was evaluated. The resulting standardization procedure consisted of two interactive parts: (i) the correlation of the flux of a test-substance with that of GT using epidermal samples from three donors and estimating the minimum, mean and maximum flux of the test compound and (ii) quantitation of the transepidermal permeation of the test compound with those standardized epidermal preparations by calculating the GT standard coefficient defined by the slope of the line derived from the relation between GT flux units and the corresponding flux from the test compound.

It is generally accepted that the stratum corneum is the major barrier for the transdermal diffusion of drugs (Scheuplein 1965). Its thickness varies among body sites (Holbrok & Odland 1974) and also with age and sex of the donor (Winkelmann 1969). As it is difficult to isolate stratum corneum, in most cases the whole epidermis is used for in-vitro testing of the skin permeation of drugs (Michaels et al 1975).

Intra-individual regional variations in transepidermal drug permeation have been reported for scopolamine (Shaw et al 1980) and glyceryl trinitrate (GT) (Karim 1981), and inter-individual differences for phenol, methanol, caffeine and aspirin (Southwell et al 1984). Although we also found wide variations in the permeability of GT through human abdominal epidermis taken from different individuals, in groups of different ages (<60 and >60 years) the mean values were in the same range (15·9 and 17·4 μg cm^{-2} h^{-1}), whereas the variance was higher in the elderly (Langguth et al 1986).

As intra-individual variations are high, a large number of skin samples must be investigated to determine the magnitude of the flux. This is necessary, since it is not known whether the epidermis to be used is highly or moderately permeable, or impermeable. Therefore, we have tried to develop a procedure that permits reduction in the number of single experiments without reducing accuracy. This may be possible by means of a standardization of epidermal preparations relative to their permeability for a standard compound. Therefore, an attempt has been made to establish whether a relation exists between the flux of a compound of high permeability such as GT, which could be used as a standard, and the fluxes of other compounds.

* Correspondence.

MATERIALS AND METHODS

Chemicals

GT–lactose trituration (1 : 10) was kindly supplied by Ciba-Geigy (Wehr, FRG), frusemide by Stada AG (Dortelweil, FRG), ethacrynic acid by MSD (Munich, FRG) and trospium chloride by Pfleger (Bamberg, FRG). Caffeine, ephedrine and benzoic acid were obtained from Synupharm (Barsbüttel, FRG).

All solvents were of analytical grade and obtained from E. Merck (Darmstadt, FRG).

Analytical instruments

HPLC analyses were performed with a P.E. LC 601 chromatograph (Perkin Elmer, Überlingen, FRG), Knauer columns 12·5 × 0·4 cm (Knauer, Berlin, FRG) filled with LiChrosorb RP-8 (5 μm) for all compounds except for ethacrynic acid (where the column length was 24 cm and the column filled with LiChrosorb RP-18), a DuPont variable wavelength

Table 1. Mobile phases for the HPLC analyses of different compounds.

Compound	Mobile phase
Glyceryl trinitrate	Distilled water–methanol (65 : 35, v/v)
Caffeine	Distilled water–methanol (65 : 35, v/v)
Ephedrine	Distilled water–acetonitrile–phosphoric acid 85% (65 : 35 : 2, v/v) containing 1 g n-decylsulphate per litre
Benzoic acid	Distilled water–methanol–formic acid (50 : 50 : 0·1, v/v)
Trospium chloride	0·01 M sodiumheptylsulphonate solution in water–acetonitrile–phosphoric acid 85% (50 : 50 : 0·15, v/v)
Ethacrynic acid	Phosphate buffer pH 3–acetonitrile (40 : 75, v/v)

detector (DuPont, Wilmington DE, USA) and a Linseis recorder (Linseis, Selb, FRG).

Quantitative thin-layer chromatographic analyses were performed with a chromatogram-spectrophotometer KM3 (Zeiss, Oberkochen, FRG).

Chromatographic conditions

Trospium chloride, caffeine, ephedrine, benzoic and ethacrynic acids were assayed by high performance liquid chromatography. The HPLC analyses were at ambient temperature (20 °C). Mobile phases, flow rates, injection volumes, wavelengths for UV-detection and retention times are listed in Tables 1 and 2.

TLC conditions (frusemide)

For the quantitative determination of frusemide, its intrinsic fluorescence was measured on the TLC plate after chromatography. Stationary phase: TLC plates precoated with silica gel 60 (Merck). Mobile phase: chloroform–methanol–acetic acid (90 : 5 : 8, v/v). Applied volume: 40 µl. R_F-value: 0·3. Detection wavelengths: excitation 265 nm, emission 436 nm.

Table 2. Chromatographic conditions for the HPLC analyses of different compounds.

Compound	Retention time (min)	Detection wavelength (nm)	Injection volume (µl)	Flow rate (ml min^{-1})
Glyceryl trinitrate	29	220	100	0·6
Caffeine	20	273	50	0·6
Ephedrine	35	220	50	1·2
Benzoic acid	21	248	50	0·6
Trospium chloride	35	220	50	1·2
Ethacrynic acid	3·5	280	25	1·1

Permeation studies

Skin permeabilities were measured in glass permeation cells at 32 °C. A piece of epidermis was mounted between two compartments filled with aqueous solutions, one containing drug solution (donor solution) and the other drug-free solution. The volume of the donor compartment was 11 ml, and the receptor compartment, 18 ml. Both compartments were stirred with bar magnetic stirrers at 60 rev min^{-1}. The area of skin between the compartments was 3·14 cm^2. The amounts of drug reaching the receptor compartment were measured after 1, 2, 3, 4, 7, 12, 24, 36, and 48 h.

Skin was obtained from the abdomens of white, Caucasian males and females at autopsy 8–24 h after death. It was quickly frozen in dry ice and stored at −20 °C according to Harrison et al (1984). Epidermis was prepared according to the method of Scheuplein (1965). Each epidermal sample was used once.

Variance of flux determinations in skin samples

The variance of the flux through the abdominal skin of one individual was tested with three compounds: GT, ethacrynic acid, caffeine. The standard deviation was investigated with larger abdominal skin samples from which several epidermal pieces (≥3) could be obtained.

Inter-individual variance of skin fluxes. To determine the inter-individual variance of the skin fluxes of the various compounds for each substance, flux measurements with epidermal membranes from at least six different individuals were made. In this case the flux was determined once for each individual membrane. Donor solutions, receptor solutions, drug amount in the donor compartment and the pH value of the donor solutions are listed in Table 3.

Standardization experiments. For the standardization experiments considerably larger epidermal sam-

Table 3. Donor and receptor solutions and drug amounts in the donor compartment.

Compound	Donor solute	pH donor solution	Amount of drug in donor (ng ml^{-1})	Receptor-solute
Glyceryl trinitrate	Water	Neutral	2·0	Water
Caffeine	Water	6·1	10·0	Water
Benzoic acid	Water	2·8	10·0	Water
Ephedrine	Buffer pH8–NaOH	8·0	10·0	Water
Frusemide	Ethanol–water 50 : 50, g/g	3·4	10·0 (suspension)	0·4% albumin in buffer pH 7·
Trospium chloride	Water	Neutral	10·0	Water
Ethacrynic acid	Water	3·8	10·0	Water

ples (about 28 cm^2 in area) were needed. First the GT flux of an epidermal sample was determined once, and afterwards the experiment was performed twice with the test compound. The relation between the flux of GT and the test compound was studied over a range of at least 10–15 GT-flux units.

Data analysis

For each sampling point, the flux was calculated in μg cm^{-2} h^{-1} according to Michaels et al (1975). For the period when the flux was constant, mean values were calculated for each individual. Assuming normal distribution of the fluxes, linear regression analysis was used to calculate the relation between the GT flux units and the corresponding fluxes of the test compounds.

The slope of the straight line was defined as the 'standard coefficient' (SC). When molar concentrations are used, the slope equals the 'molar standard coefficient', which can also be calculated from SC and the molecular weights of the compounds according to the equation:

$$\text{molar SC(GT)} = \text{SC(GT)} \times (MG_{GT}/MG_X)$$

This coefficient should be 1 for GT if it is chosen as the standard. It should be <1 for compounds with lower fluxes than GT, and >1, if a compound permeates better than GT.

RESULTS

Variability studies

Variance of flux determinations per abdominal sample. The results from the repetitive measurements of the fluxes through the abdominal epidermis of one donor are given in Table 4. The standard deviations are always <5%.

Table 4. Standard deviations (in percent of the mean) if several epidermal samples were obtained from abdominal skin of one donor.

Tested compounds	No of replicate determinations	s.d. %
Glyceryl trinitrate	6	2·9
	5	3·1
	3	4·1
Ethacrynic acid	7	3·8
	3	4·9
Caffeine	4	2·0
	4	4·1

Inter-individual variability. The transepidermal permeation of various compounds with different properties (acid/base-character, lipophilicity, molecular weight) was tested. The GT variability data were taken from Langguth et al (1986). The results of these studies are summarized in Table 5. The variability and distribution of the values are illustrated in Fig. 1. From the data, it becomes clear that the range of variation is high for each of the compounds. Even for substances such as ethacrynic acid, benzoic acid and ephedrine which have good permeability, the lowest and the highest flux differ at least by the factor 2·8 (for benzoic acid with a sample size of 6 only). For GT fluxes this factor was 9. The standard deviation of the GT fluxes was 42·9% (n = 31). The highest standard deviation was found with the quaternary compound trospium chloride, which had a very low flux (Table 5). For frusemide the lowest and highest flux differ by a factor of 6·25.

Table 5. Variability of the transepidermal fluxes of the investigated compounds (the fluxes are given as μg cm^{-2} h^{-1}).

Compound	Number of different skin samples	Arithmetical mean $\bar{x}$	Standard deviation s.d (%)	Median $\tilde{x}$	Range (x_{max}–x_{min})
Glyceryl trinitrate	25	16·5	42·9	15·3	32·5 (36·9–4·1)
Frusemide	7	0·55	69·9	0·43	0·84 (1·0–0·16)
Trospium chloride	13	0·20	118·4	0·063	0·78 (0·78–0)
Ephedrine	6	6·94	53·1	6·43	9·82 (11·9–2·08)
Caffeine	6	1·69	73·9	1·32	3·37 (3·96–0·59)
Benzoic acid	6	13·88	38·8	14·48	13.0 (20·3–7.3)
Ethacrynic acid	6	13.58	88·5	8·02	26·0 (31·2–5·2)

Correlations

Epidermal membranes from at least five different donors were tested to investigate the relation between the GT flux and the flux of a compound X, the test compound. Ephedrine, frusemide, caffeine, benzoic acid and trospium chloride were included as

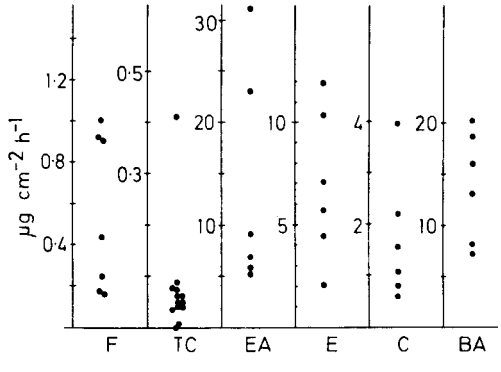

FIG. 1. Fluxes of several compounds through human epidermis. Each of the data points characterizes the mean flux through the epidermal membrane of one individual, given as μg cm^{-2} h^{-1}. Key: F, frusemide; TC, trospium chloride; EA, ethacrynic acid; E, ephedrine; C, caffeine; BA, benzoic acid.

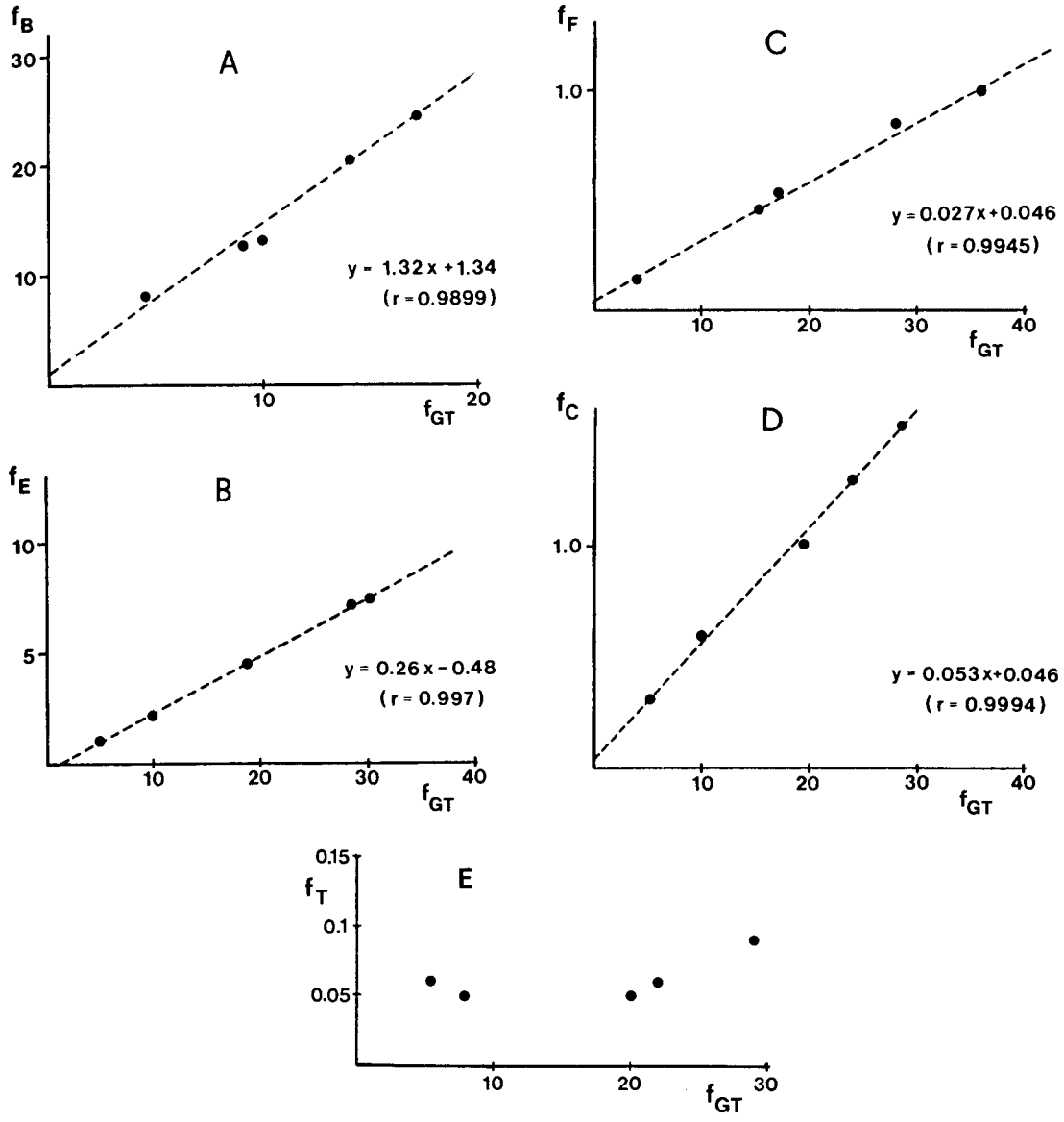

FIG. 2. Correlations between the flux (f) of the tested compounds (a: benzoic acid (B), b: ephedrine (E), c: frusemide (F), d: caffeine (C)) and the GT flux at epidermal preparations with different GT permeability; e: correlation between the fluxes of the trospium chloride and glyceryl trinitrate (y = 0·0011x + 0·0438; r = 0·6979).

test compounds. The relations found are shown in Fig. 2 A–E. With ephedrine, frusemide, caffeine and benzoic acid a good linear relation could be detected. The correlation coefficients were always >0·9.

The molar GT standard coefficients (molar SC(GT)) for these compounds are 0·34 for ephedrine, 0·02 for frusemide, 0·06 for caffeine and 2·40 for benzoic acid. The GT standard coefficient of GT itself was proved to be 1·0.

In contrast with these results, the trospium fluxes did not correlate with GT (Fig. 2E), but it is a quaternary compound with very low intrinsic transdermal fluxes.

CONCLUSIONS

Our investigations demonstrate that inter-individual variations in transepidermal permeation are high. Therefore, to determine the mean and the minimum

flux of a compound through human epidermis exactly, it was necessary to perform many experiments with different skin samples.

To minimize the experimental effort, standardized epidermal preparations can be used. The procedure consists of the following steps: (i) determination of the GT flux range (mean, minimum and maximum) for the available apparatus and the experimental conditions, (ii) determination of the flux of GT and another compound for each skin sample, using (3–4) larger abdominal skin samples, (iii) calculation of the relation between the GT flux and the flux of the test-compound and if this is linear, (iv) estimation of the flux range of the test compound, (v) determination of the molar standard coefficient. As standard substances, compounds that permeate well, and which can easily be quantified in the receptor solutions (as e.g. GT or clonidine) are useful.

If GT standardized epidermal preparations are used, a range of about 15 GT flux units should be covered by at least three skin samples. In the case of GT, 1 flux unit is 1 $\mu g\ cm^{-2}\ h^{-1}$. If another standard is used, the variability of the flux has to be determined in a sufficient number of cases before starting the experiment.

The procedure for GT standardization is illustrated and summarized in Fig. 3.

For GT a mean flux of 16·9 $\mu g\ cm^{-2}\ h^{-1}$ (number of skin donors = 31) was found under the defined experimental conditions. An approach to the mean flux of the test compound would be the f_X value at 16·5 GT units, the minimal flux would be at 4·1 GT units and the maximum flux at 36·9 GT units. Thus it is possible to predict the corresponding range of the minimum, mean and highest fluxes of a test compound through epidermal membranes. On the other hand, the calculated molar GT standard coefficient allows direct comparison of the flux of a test compound with GT or other compounds under defined experimental conditions.

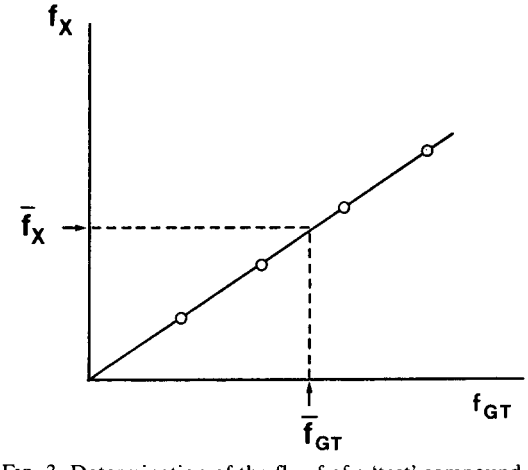

FIG. 3. Determination of the flux f of a 'test' compound X using glyceryl trinitrate as standard compound (under defined experimental conditions).

If the correlation coefficient between the GT flux and the flux of an unknown compound is lower than 0·8, the present method cannot be used, as with trospium chloride. Quaternary compounds like trospium chloride need further investigation regarding standardization.

An explanation for the different behaviour of trospium chloride can be seen in the fact that this quaternary compound is very hydrophilic and primarily permeates skin through hydrophilic pores. On the other hand, compounds like GT or benzoic acid mainly use the transcellular route to cross the epidermis in large amounts. Thus, different mechanisms of transepidermal permeation of GT and trospium chloride could be an explanation for the lack of a good correlation.

Acknowledgement

This work was supported by a grant of Stada AG, Dortelweil (FRG).

REFERENCES

Harrison, S. M., Barry, B. W., Dugard, P. H. (1984) J. Pharm. Pharmacol. 36: 261–262

Holbrok, K. A., Odland, G. F. (1974) J. Invest. Dermatol. 62: 415–422

Karim, A. (1981) 8th Int. Congr. Pharmacol., Tokyo (presentation)

Langguth, P., Spahn, H., Mutschler, E. (1986) Pharm. Res. in press

Michaels, A. S., Chandrasekaran, S. K., Shaw, J. E. (1975) Am. J. Chem. Eng. 5: 985–996

Scheuplein, R. J. (1965) J. Invest. Dermatol. 45: 334–346

Shaw, J. E., Taskowich, L., Chandrasekaran, S. K. (1980) in: Current Concepts in Cutaneous Toxicity, Academic Press, New York, pp. 127–133

Southwell, D., Barry, B. W., Woodford, R. (1984) Int. J. Pharm. 3: 299–309

Winkelmann, R. K. (1969) Br. J. Derm. 81: Suppl. 4, 11–22