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Statistical testing of drug accumulation in skin tissues by linear regression versus contents of stratum corneum lipids

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Dedicated to Professor Dr B.C. Lippold, Düsseldorf, on the occasion of his 60th birthday.

Abstract

This investigation is a contribution to standardization in in vitro drug penetration measurements using excised human skin and to statistical treatment of the observations. The wide variations observed in measurements of drug accumulation in and drug permeation through the stratum corneum are caused not only by analytical errors but also by the variability of the horny layer lipid composition. The last-mentioned systematic influence can be compensated for by stepwise (multiple) linear regression using the contents of the main lipid classes as independent variables. In consequence, the S.E. of estimate given by the regression calculation is lower than the S.E. of the means of the observations. Significant differences in drug quantities accumulated in skin tissues (stratum corneum and dermis) are sensitively detected by Chow's *F*-test of structural change. Accumulation data of flufenamic acid and hydrocortisone penetrated from different bases are given as examples. The calculation mode is exemplarily explained and discussed. The results of the test for structural change, two-independent-groups *t*-test and paired-samples *t*-test are compared. The *F*-test of structural change proves to be a helpful statistical method suitable to the assessment of biopharmaceutical quality parameters and to measurements using biological materials.

Keywords: Drug accumulation in stratum corneum and dermis; Influence of skin lipid composition and vehicles; Multiple linear regression; Significance of differences in statistical tests; Chow's test of structural change; Flufenamic acid; Hydrocortisone

1. Introduction

Skin penetration and permeation measurements are highly variable. Reviewing the literature and referring to their extensive experimental data, Williams et al. (1992) and Cornwell and Barry (1995) reported that drug permeability coefficient measurements are expected to have a mean intra-individual coefficient of variation of approximately 40% and a mean inter-individual coefficient of variation of about 70% (Southwell et al., 1984). These experiences correspond to the results of Wenkers and Lippold (1999) and to our

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own experimental in vitro and in vivo investigation. The high variability is not only a consequence of analytical errors but also of great differences in the biological material, especially in composition and structure of the lipid domain of the stratum corneum (Elias et al., 1981; Elias, 1990; Wertz et al., 1992; Norlen et al., 1999; Marjukka Suhonen et al., 1999). These parameters vary with body region and from person to person (Lampe et al., 1983a; Rougier et al., 1987; Wertz et al., 1992). Law et al. (1995) observed an inverse correlation between horny layer permeability and content of ceramides according to their structure-forming properties. Thus, systematic influences of the lipid content and composition on drug accumulation and permeation are suggested. Seizing these effects is the aim of the present investigation, in order to improve the detection of significant differences in biopharmaceutical tests.

2. Materials and methods

2.1. Materials

2.1.1. Standard substances

Flufenamic acid (Kali Chemie Pharma, Hannover, Germany); hydrocortisone (Caelo, Hilden, Germany); cholesterol, cholesterol oleate, glycerol trioleate, oleic acid, ceramide 3, ceramide 4, standard lipids no. 178–4 consisting of 25% cholesterol, cholesterol oleate, glycerol trioleate, and oleic acid (Sigma, Deisenhofen, Germany).

2.1.2. Vehicles

Eutanol® G: 2-octyl dodecanol (Henkel, Düsseldorf, Germany); semisolid triglycerides: Softisan® 378 (Hüls, Witten, Germany); white petrolatum; nonionic hydrophilic cream (German Pharmacopoeia): comprising 5 g polysorbate 60, 10 g cetostearyl alcohol, 10 g glycerol (85%), 25 g white petrolatum, 50 g water; wool alcohols cream (Reynolds, 1996): comprising 0.25 g cetostearyl alcohol, 3 g wool wax alcohols, 46.75 g white petrolatum, and 50 g water.

2.1.3. Solvents

Solvents were of analytical purity or HPTLC or HPLC grade (Merck, Darmstadt, Germany).

2.1.4. Copper sulfate–phosphoric acid reagent

$CuSO_4 \cdot 5\ H_2O$ (10 g) and 10 g H_3PO_4 (85%) dissolved in 80 g water.

2.1.5. HPTLC equipment

HPTLC silica gel plates 20×10 cm without indicator (Merck, Darmstadt, Germany); Linomat IV with 100 μl syringe, horizontal HPTLC chamber 10×20 cm (Camag, Berlin, Germany); dipping chamber for HPTLC plates, 'Tauchfix' (Baron, Insel Reichenau, Germany); Thermoplate S, densitometric scanner CD 60 with integration program, version 3.0 (Desaga, Heidelberg, Germany).

2.1.6. HPLC equipment

HPLC pump L 6220, HPLC uv–vis detector L 4250, HPLC autosampler 655 A 40, HPLC interface D 6000 A (Merck-Hitachi, Darmstadt, Germany); on-line solvent degasser ERC-3315 (ERC, Alteglofsheim/Regensburg, Germany).

2.2. Excised human skin

Immediately after necessary surgery, human skin samples excised from the abdomen, thigh, calf, and mamma of different subjects were carefully freed from subcutaneous fat tissue. Samples were stored at -26°C for a maximum of 8 weeks enclosed in welded polyethylene bags wrapped in aluminium foil.

2.3. Analysis of stratum corneum lipids

2.3.1. Separation of stratum corneum from viable epidermis (according to Lampe et al. (1983b) and Wertz et al. (1992)) and lipid extraction

Excised human skin consisting of epidermis and dermis was cut by means of a punch so that cylindrical pieces (diameter 15 mm) were obtained. The samples were incubated with 0.1% trypsin solution in PBS buffer at 30°C for 24 h. The separated layer consisting of stratum corneum and epidermis was rinsed with PBS

buffer and water and then treated with trypsin solution at 30°C for a further 2 h. The horny layer was put on a teflon foil (cleaned with chloroform–methanol (2:1)) and dried in a desiccator over silica gel at room temperature.

The stratum corneum piece was weighed and extracted by shaking twice with 5 ml chloroform–methanol (2:1) at room temperature for 2 h followed by ultrasound treatment for 10 min. The extract was filtered (teflon filter disc, mean pore diameter 0.2 μm; Sartorius, Göttingen, Germany), evaporated to dryness, and dissolved in chloroform–methanol (2:1). If not used immediately, this solution was stored at −25°C.

2.3.2. Lipid separation and quantification

HPTLC separation was performed on silica gel plates (mean particle diameter 6 μm) at room temperature using the following solvent systems:

1. For the analysis of ceramides, free fatty acids, and sterols: chloroform–methanol–glacial acetic acid (190:9:1, v/v/v).
2. For the analysis of sterols, fatty acids, and other acidic substances (e.g. flufenamic acid): *n*-hexane–ether–glacial acetic acid (80:20:10, v/v/v).
3. For the analysis of triglycerides, sterol esters, and *n*-alkanes: first development with *n*-hexane–ether (80:20, v/v) up to a solvent front of 4 cm above the start point, and after drying, second development with petroleum ether up to a solvent front of 8 cm.
4. For the analysis of triglycerides and sterol esters: first development with *n*-hexane–ether (40:16, v/v) up to a solvent front of 4 cm above the start point, and after drying, second development with petroleum ether up to a solvent front of 8 cm.

The lipid substances were detected by treatment with copper sulfate–phosphoric acid reagent (Touchstone et al., 1983) and identified by authentic samples. As the detection of lipids with this reagent proceeds under charring which strongly depends on the experimental conditions, standard and horny layer lipids have to be subjected to identical treatment: the plates were dipped for 1 s into the reagent by means of a dipping chamber and then heated to 110°C for 5 min and to 160°C for 15 min. The lipids were quantified by densitometry in reflection mode ($\lambda = 326$ nm) using an integration program. Linear calibration was performed on at least eight standard substances (mass range 10–100 ng).

For practical reasons the lipid contents (Table 3) are given as absolute amounts of the lipid classes contained in 1 mg of dehydrated horny layer (used in flufenamic acid penetration experiments) or in a specified volume of the stratum corneum (= stripped volume = strip thickness times base area of 1.8 cm^2; used in hydrocortisone penetration experiments).

2.4. In vitro drug penetration

Excised human skin consisting of epidermis and dermis was cut by means of a punch (diameter 22 mm) so that cylindrical pieces were obtained. The skin sample was inserted into the cavity of a teflon block with a filter disk inlay saturated with Ringer solution. The semisolid drug preparation was filled into a teflon chamber (diameter 18 mm, depth 3 mm) and then pressed onto the Stratum corneum by a mass of 500 g. This equipment (Blasius, 1985), which was placed within a closed chamber, was equilibrated at 30°C. At the end of the penetration time, the ointment chamber was removed, the skin surface thoroughly cleaned with cotton wool, and then the horny layer stripped immediately.

2.4.1. Flufenamic acid preparations

Flufenamic acid (10%) compounded with petrolatum, petrolatum + eutanol (9:1), semisolid triglycerides or semisolid triglycerides + eutanol (9:1); penetration time 2 h.

2.4.2. Hydrocortisone preparations

Hydrocortisone (1%) compounded with semisolid triglycerides, white petrolatum, nonionic hydrophilic cream or wool alcohols cream; penetration time 3 h.

2.5. Stripping the stratum corneum

Skin samples from the penetration experiments were stripped with adhesive tape (Tesafilm plus,

5652 AF 60, Beiersdorf, Hamburg, Germany) using a self-made device and the procedure developed by Borchert (1994). As the drug amounts in the strips decrease with increasing depth of the horny layer, several strips were combined to form a single sample so that the amounts of drug to be measured were sufficiently in excess of the determination limit (defined according to Funk et al. (1985)). The quantities given in Table 3 are the sums of the contents of the fractions.

2.6. Slicing the dermis

After stripping the stratum corneum, the remaining tissue was frozen by a stream of carbon dioxide and sliced to a thickness of 25 μm parallel to the surface by means of a cryo-microtome. The drugs were extracted (flufenamic acid by chloroform–methanol, 2:1; hydrocortisone by methanol–water, 3:2, adjusted with phosphoric acid to pH 3) and quantified by means of the methods described below.

2.7. Drug quantities in horny layer and dermis

The measurements of the extent of drug penetration and lipid analyses were performed separately on different parts of the same skin piece. The observed amounts of drugs and eutanol given in Tables 1 and 2 are related to the diffusion area, i.e. the drug content in the volume of the stratum corneum or dermis over a cross section of 1.8 cm^2.

2.7.1. Quantitative determination of flufenamic acid

Flufenamic acid was extracted by shaking the horny layer strip fractions with 10 ml diethylether for 30 min. After filtration (Teflon filter, mean pore diameter: 0.2 μm. Sartorius, Göttingen, Germany), the remaining tapes were washed with ether and the combined extracts evaporated to dryness. The residue dissolved in methanol was used for HPTLC. After developing with chloroform–methanol (24:5, v/v), the chromatogram was densitometrically scanned using the absorption mode at $\lambda = 283$ nm (flufenamic acid concentration > 20 ng/spot) or the fluorescence mode (drug concentration < 50 ng/spot; activation by treatment with 2% $ZrOCl_2 \cdot 8H_2O$ reagent in methanol and heating to 110°C for 10 min, measurement at $\lambda = 366$ nm using a 400 nm cut-off filter; Poethke et al. (1970)). Extract samples and 4–5 standard samples having different drug amounts were run on each plate and used for evaluation by linear regression. The same HPTLC plate can be quantified firstly in the absorption mode and then in the fluorescence mode.

2.7.2. Quantitative determination of hydrocortisone

Adhesive tape stripping was performed as described above. The strips combined to form several fractions were extracted with 2 ml methanol–water (6:4, v/v, adjusted with phosphoric acid to pH 3) by shaking in a waterbath at 40°C for 2 h. After filtration, the hydrocortisone was estimated by HPLC: Injected volume: 50–100 μl, solvent: methanol–water as described above, photometric detection and determination by UV absorption at 245 nm (peak area) within the linear concentration range (0.25–3 μg/ml).

2.8. Statistical calculations

Statistical calculations were performed by means of the PC program SPSS for Windows, version 8.0.

3. Results and discussion

3.1. Drug penetration experiments

In vitro drug penetration was performed in the infinite dose mode using excised human skin pieces ('Saarbrücker model') which are not in contact with a voluminous receptor like buffer solution or another fluid, in order to avoid unnatural swelling and to minimize bacterial destruction during the experiments. Under this condition the receptor function is served by the dermis. Because of its limited capacity for dissolving the diffusants, the penetration period has to be chosen in such a manner that the permeant concentration in the horny layer is near to the maximum, whereas the concentration in the dermis is low at

Table 1
Accumulation of flufenamic acid (flu) and eutanol® (eu) in stratum corneum and dermis after 2 h penetration from various vehicles: observed values, arithmetic means (μ_a), and S.D.[a]

Skin sample	Stratum corneum				Dermis			
	Flu (nmol) from pet	Flu (nmol) from epet	Flu (nmol) from stri	Flu (nmol) from estr	Flu (nmol) from pet	Flu (nmol) from epet	Flu (nmol) from stri	Flu (nmol) from estr
Observations								
A.1	20.84	33.10	168.50	142.40	40.18	55.11	51.84	54.54
B.1	67.95	39.01	142.60	120.30	22.90	18.17	29.16	26.92
B.2	45.23	60.84	104.90	138.10	26.92	18.21	26.92	26.92
B.3	53.30		107.70				29.01	
C.1	63.75	49.53	136.10	89.99	18.35	20.13	25.42	51.66
C.2	54.40	31.89		133.80	23.93	20.30	25.60	52.20
C.3	54.33						25.35	
D.1	117.60	166.30	175.50	137.20	35.70	33.35	38.79	29.97
D.2	85.91	160.30	137.50	130.60	33.81	35.38	36.02	
D.3				164.60				
Means								
μ_a	62.59	77.28	139.0	132.1	28.83	28.66	32.01	40.37
±S.D. (%)	±27.08 (43.3)	±59.63 (77.2)	±26.98 (19.4)	±21.19 (16.0)	±7.89 (27.4)	±13.72 (47.8)	±8.85 (27.7)	±13.70 (33.9)
		Eu (μg) from epet		Eu (μg) from estr				
Observations								
A.1		7.73		44.65				
B.1		7.03		25.50				
B.2		9.59		30.38				
C.1		12.21		25.23				
C.2		6.58		38.76				
D.1		29.33		25.41				
D.2		29.67		29.06				
D.3				40.27				
Means								
μ_a		14.59		32.41				
±S.D. (%)		±10.36 (71.0)		±7.70 (23.8)				

[a] Vehicles: pet, white petrolatum; epet, white petrolatum + eutanol® (9:1); stri, semisolid triglycerides; estr, semisolid triglycerides + eutanol® (9:1).

Table 2
Accumulation of hydrocortisone (hc) in stratum corneum and dermis after 3 h penetration from various vehicles: observed values, arithmetic means (μ_a), and S.D.s[a]

Skin sample	Stratum corneum				Dermis			
	Hc (nmol) from nhc	Hc (nmol) from stri	Hc (nmol) from pet	Hc (nmol) from wac	Hc (nmol) from nhc	Hc (nmol) from stri	Hc (nmol) from pet	Hc (nmol) from wac
Observations								
G.1	20.37	24.45	21.26	15.70	1.13	1.67	1.04	0.85
G.2	17.97	20.55	21.31	16.84	1.26	0.97	0.68	0.51
H.1	11.91	11.34	10.43	6.42	1.19	1.20	0.72	0.96
H.2	13.05	11.05	11.56	8.43	1.01	1.04	0.96	0.58
I.1		9.97				1.68		
I.2	6.84	7.66			0.58	0.58		
K.1	9.03	11.68			0.69	1.06		
K.2	11.97	13.05			0.54	0.18		
L.1	19.21	12.03						
Means of skin samples G and H								
μ_a	15.83	16.85	16.14	11.85	1.15	1.22	0.85	0.73
±S.D. (%)	±4.01 (25.4)	±6.72 (39.9)	±5.96 (36.9)	±5.19 (43.8)	±0.11 (9.2)	±0.32 (25.8)	±0.18 (20.8)	±0.21 (29.6)
Means of skin samples G, H, I, and K								
μ_a	13.02	13.72			0.91	1.05		
±S.D. (%)	±4.74 (36.4)	±5.73 (41.8)			±0.30 (33.2)	±0.51 (48.3)		
Means of skin samples G, H, I, K, and L								
μ_a	13.79	13.53						
±S.D. (%)	± 4.91(35.6)	± 5.39(39.8)						

[a] Vehicles: nhc, nonionic hydrophilic cream; stri, semisolid triglycerides; pet, white petrolatum; wac, wool alcohols cream.

that time. On the basis of preceding tests, flufenamic acid formulations have been allowed to penetrate for 2 h and hydrocortisone preparations for 3 h.

3.2. Comparison of mean quantities accumulated in skin tissues by t-tests

The values of drug amounts accumulated in the stratum corneum and dermis are summarized in Tables 1 and 2. The confidence intervals i_c of the arithmetic means (probability $P = 95\%$) are calculated by:

$$i_c = \mu_a \pm t \times s/n^{0.5} \quad (1)$$

where μ_a is the arithmetic mean, s is the S.D., n is the number of observations, and t is the t-distribution value tabulated in dependence on degree of freedom and probability. Figs. 1 and 2 illustrate the high variability of the data and the low differentiation between the formulations. Confidence intervals not overlapping at $P = 95\%$ can be detected in few cases only: flufenamic acid accumulated in the stratum corneum after penetration from petrolatum in comparison to penetration from semisolid triglycerides and semisolid triglycerides + eutanol. The arithmetic means which show S.D.s of $\pm 16 - \pm 77\%$ in the stratum

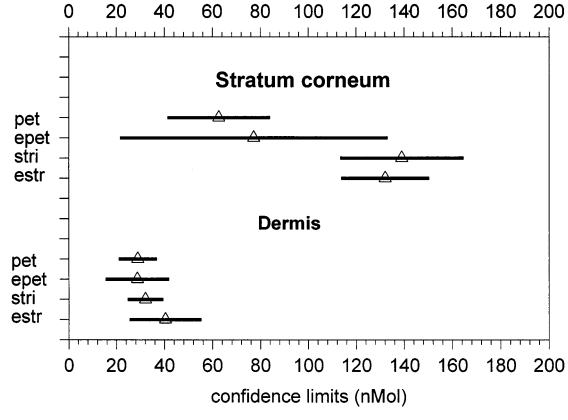

Fig. 1. Flufenamic acid accumulation in stratum corneum and dermis after penetration from several vehicles: arithmetic means with confidence intervals at $P = 95\%$. Vehicles: pet, white petrolatum; epet, white petrolatum + eutanol (9:1); stri, semisolid triglycerides; estr, semisolid triglycerides + eutanol (9:1).

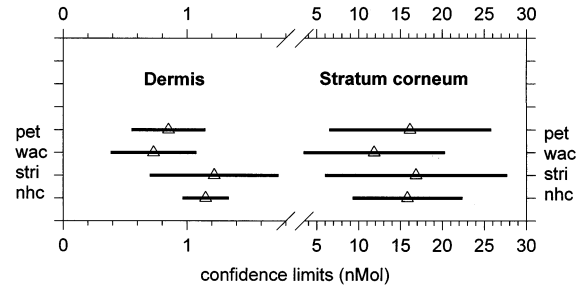

Fig. 2. Hydrocortisone accumulation in stratum corneum and dermis after penetration from several vehicles: arithmetic means with confidence intervals at $P = 95\%$. Vehicles: pet, white petrolatum; wac, wool alcohols cream; stri, semisolid triglycerides; nhc, nonionic hydrophilic cream.

corneum and of $\pm 9 - \pm 48\%$ in the dermis have been compared by the t-test with two independent groups of observations with unequal sizes and variances (Armitage and Berry, 1987; Sachs, 1997) and the results are given in Table 4a. Weir's t-test (suitable to small sample sizes $n_i \geq 3$) gives corresponding results.

Observations which are obtained from different formulations applied to skin of the same source can be paired and compared using Eq. (2):

$$t_{cal} = \frac{\delta_m \times N^{0.5}}{s_\delta} = \frac{\left(\sum \delta\right)/N}{\left(\left(\sum \delta^2 - \left(\sum \delta\right)^2/N\right)/(N \times (N-1))\right)^{0.5}} \quad (2)$$

with t_{cal} is the t value calculated, δ is the difference of two paired observations, δ_m is the mean of differences, s_δ is the S.D. of δ, and N is the number of differences.

The detection of significant differences is clearly more sensitive than with the two-independent-groups t-test as Table 4b indicates. This results from the mode not to use the observations but the differences of paired samples for the calculation of the test values t_{cal}. The stratum corneum of two skin pieces taken from the same source and used for the paired experiments has nearly equal properties so that the difference δ is rather free from influence of skin qualities on drug accumulation. This points to the dependence of drug accumula-

tion on lipid content and composition of the horny layer.

3.3. Correlation of accumulated drug quantities with content of lipids

Calculating the correlation of drug enrichment with total lipid content by linear regression results in regression equations with low correlation coefficients ($r \leq 0.4$). The correlation coefficients rise, if the correlation with the sum of the amounts of ceramides, sterols, sterol esters, and free fatty acids (triglycerides omitted) is tested, but does not show satisfying values. On the other hand, clearly visible differences of substance quantities accumulated in stratum corneum and dermis are observed after penetration of a drug from the same preparation into skin samples of various donors having different lipid patterns as is evident from Tables 1–3 (compare accumulated drug quantities in Table 1 samples A/B/D and in Table 2 samples G/I, for example). Furthermore, comparing the flufenamic acid quantities enriched in the stratum corneum of samples A–D by the least significant difference method 'LSD' (Armitage and Berry, 1987; Sachs, 1997) significantly indicates that the drug contents in D are higher than in A, B, and C. Vice versa, D is shown to have lower contents of ceramides and sterols than A, B, and C (see below). In accordance with the results of Law et al. (1995) it is suggested, therefore, that the drug amounts enriched in the tissues are related to the contents of certain lipid components; this is statistically tested for this reason. As the stratum corneum lipids are composed of a very great number of individual compounds (Elias, 1990; Wertz, 1996), the main substance classes, i.e. ceramides (cer), free fatty acids (ffa), sterols (ster), sterol esters (stest), and triglycerides (tri), were quantified as such and not in terms of their individual components.

3.4. Analysis of stratum corneum lipids

The lipid analysis used in this investigation (see Section 2.3) was based on methods developed by Lampe et al. (1983a) and Melnik et al. (1989). Recovery experiments in the absence and presence of flufenamic acid were performed using artificial lipid mixtures absorbed on filter paper (recovery:

Table 3
Composition of stratum corneum lipids of excised skin samples from several donors[a]

Skin sample	Ceramides	Sterols	Sterol esters	Free fatty acids	Triglycerides
μg Lipid/ mg desiccated stratum corneum					
A ($n=3$)	29.18 ± 5.2	20.63 ± 2.7	15.33 ± 8.7	24.50 ± 8.9	65.76 ± 2.9
B ($n=6$)	29.92 ± 3.2	19.33 ± 5.5	8.81 ± 7.9	13.88 ± 12.1	38.11 ± 16.3
C ($n=2$)[b]	29.38 ± 1.9	21.85 ± 3.4	12.70 ± 4.7	20.60 ± 2.2	183.85 ± 16.8
D ($n=5$)	22.68 ± 4.2	15.04 ± 6.9	6.46 ± 7.5	15.32 ± 9.2	136.16 ± 14.8
Mean A–D (x_{am})	26.1	18.1	9.8	17.1	123.9
μg Lipid per defined volume of stratum corneum (see text)					
G ($n=1$)[b]	32.89	14.59	8.68	36.59	147.72
H ($n=1$)[b]	41.21	16.81	16.01	24.17	44.80
I ($n=2$)[b]	43.62 ± 21.4	36.34 ± 1.4	16.04 ± 15.7	24.77 ± 22.9	137.39 ± 31.3
K ($n=3$)	43.40 ± 6.3	25.09 ± 13.3	15.37 ± 3.8	21.16 ± 1.8	49.93 ± 10.4
L ($n=1$)[b]	29.65	28.99	7.16	32.27	51.38
Mean G–L (x_{am})	38.15	24.36	12.65	27.79	86.24
Mean G–K (x_{am})	40.28	23.21	14.03	26.67	94.96
Mean G–H (x_{am})	37.05	15.70	12.35	30.38	96.26

[a] n = Number of tests, S.D. of lipid analysis in %, x_{am} = mean content of different skin samples, x_{am} is used for the calculation of ava.

[b] Because of insufficient quantity of skin material from the respective donor.

98–102% of saturated and unsaturated fatty acids, cholesterol, cholesterol oleate, and triglycerides; 91–93% of ceramides). Furthermore, the analysis of ceramides was controlled by stocking up the horny layer with known amounts of these substances (recovery: 94–95%). The lipid composition of skin samples from different donors show considerable variation (Table 3), especially with respect to the triglyceride contents (38–195 μg/mg dehydrated stratum corneum, corresponding to 35–80% of total lipids). Contamination from subdermal fat tissue during and after skin excision cannot be ruled out (Lampe et al., 1983a; Hedberg et al., 1988) and may cause an abnormally high triglyceride content in the horny layer. Investigations which use excised skin pieces must, therefore, address this problem. Wertz et al. (Wertz et al., 1987; Wertz, 1996) have reported that triglycerides are not genuine components of the stratum corneum lipids. Triglycerides partly originate from sebum and are often also applied to the skin surface as ingredients in creams or ointments. Because of the wide variation in triglyceride contents, it is very difficult to compare the relative contents of each lipid group related to the total lipid content in several skin samples. We, therefore, prefer to use the absolute amounts of lipids contained in 1 mg of dehydrated horny layer or in a specified volume of the stratum corneum (stripped volume = strip thickness times base area of 1.8 cm^2).

The contents of the respective lipid classes in skin samples A–D were compared by the Scheffé test for equality of mean values with paired means at the 5% significance level (Armitage and Berry, 1987; Sachs, 1997). Significant differences were established for:

ceramides and sterols in sample D in comparison to samples A, B, and C,
sterol esters in sample D in comparison to A and C which also differ from B,
fatty acids in samples B and D in comparison to A and C, and
triglycerides in samples C and D in comparison to A and B.

In view of the results, these skin samples were considered as suitable for testing the correlation between drug accumulation and lipid composition of the stratum corneum.

3.5. Multiple linear regression

Eq. (3) represents the general form of a multiple linear regression equation:

$$m_{drug} = b_0 + b_1 \times m_1 + \cdots + b_n \times m_n \quad (3)$$

where m_{drug} is the drug quantity (nmol) accumulated in the tissue, b_n is the regression coefficients, m_n is the amounts (μg) of the respective lipid class per mass (or defined volume) of the horny layer (see Section 2.3).

The five lipid classes mentioned above were offered for inclusion into the regression equation as independent variables and selected by the stepwise calculation mode considering the null hypothesis (H_0) $b_1 = b_2 = \cdots = b_n = 0$: regression coefficients which were shown not to be equal to zero at a preset significance level were included in the equation. They may be excluded later if they exceed the significance criteria for exclusion in the course of the calculation. In this manner, several regression equations may be obtained with increasing inclusion and exclusion criteria. The 'best' equation can be selected by regarding the (multiple) correlation coefficients (r), the S.E.s of estimate, and the residues as criteria. For example, the following regression equations describing drug accumulation in the stratum corneum were obtained from penetration experiments with skin samples from different human subjects.

Formulation: flufenamic acid/petrolatum; skin samples: A, B, C, D; inclusion/exclusion criteria $\leq 0.05/\geq 0.07$:

$$m_{flu} = 261 - 7.06 \times m_{cer} \quad (r = -0.797) \quad (4a)$$

inclusion/exclusion criteria $\leq 0.20/\geq 0.22$:

$$m_{flu} = 164 - 2.00 \times m_{cer} - 6.28 \times m_{stest} + 0.174 \times m_{tri} \quad (r = -0.927) \quad (4b)$$

Formulation: flufenamic acid/petrolatum + eutanol (9:1); skin samples: A, B, C, D; inclusion/exclusion criteria $\leq 0.05/\geq 0.07$:

$$m_{flu} = 553 - 17.3 \times m_{cer} \quad (r = -0.974) \quad (5a)$$

inclusion/exclusion criteria $\leq 0.10/\geq 0.12$:

$$m_{flu} = 572 - 16.2 \times m_{cer} - 2.70 \times m_{ffa} \quad (r = -0.990) \tag{5b}$$

Formulation: hydrocortisone/semisolid triglycerides, skin samples: G, H, I, K, L; inclusion/exclusion criteria $\leq 0.05/\geq 0.07$:

$$m_{hc} = -5.43 + 0.695 \times m_{ffa} \quad (r = 0.796) \tag{6a}$$

inclusion/exclusion criteria $\leq 0.10/\geq 0.12$:

$$m_{hc} = 5.35 + 0.535 \times m_{ffa} - 0.269 \times m_{ster} \quad (r = 0.889) \tag{6b}$$

Formulation: hydrocortisone/nonionic hydrophilic cream; skin samples: G, H, I, K, L; inclusion/exclusion criteria $\leq 0.05/\geq 0.07$:

$$m_{hc} = 44.5 - 0.796 \times m_{cer} \quad (r = -0.931) \tag{7a}$$

inclusion/exclusion criteria $\leq 0.10/\geq 0.12$:

$$m_{hc} = 45.6 - 0.721 \times m_{cer} - 0.180 \times m_{ster} \quad (r = -0.971) \tag{7b}$$

Depending on the inclusion/ exclusion criteria, the calculation procedure included up to four independent variables into the regression equations. But because of the sample size, we did not use more than two regression coefficients in general. Although the series of experiments with flufenamic acid mentioned above was performed with the same set of skin samples for both the drug preparations, different variables were included into the Eqs. (4b) and (5b). The same is true for the series of hydrocortisone penetration measurements (Eq. (6a)/Eq. (7a) and Eq. (6b)/Eq. (7b)). The equations, therefore, do not have general validity for a particular drug tested on a certain set of skin samples. The type of formulation, for example, is a parameter which can have an effect on the choice of the regression coefficients. Changing the combination of skin sample sets also results in different equations. Hence, comparing several drug formulations by skin penetration experiments evaluated by multiple linear regression requires a set of skin pieces from several subjects, and all the drug formulations tested have to be applied to all the samples of the skin assortment.

Regression equations comprising different variables have different coordinate systems so that the preconditions for comparison by confidence intervals are not met. The test of structural change according to Chow (Schneeweiß, 1990; Greene, 1993) can solve this problem: The observations of two test series which are to be compared (two formulations with different vehicles, for example) are joined to a common data set and submitted to a common regression calculation. The hypothesis is to test that the common regression equation is equivalent to both the subsets of the data, i.e. that all the regression coefficients are equal for both the subsets. We proceed as follows:

1. The common data set of the two test series to be compared is subjected to multiple linear regression calculations in the stepwise mode.
2. Regression calculations including the variables established by point 1 are performed separately with the observations of both the subsets.
3. The F statistic is computed by Eq. (8) using the residuals (sum of squares about regression) which result from the three regressions according to point 1 and 2. The calculated F-value (F_{cal}) is compared to F distribution values tabulated (F_{tab}).

$$F_{cal} = \frac{[1/m] \times \left[\sum u_0^2 - \left(\sum u_1^2 + \sum u_2^2\right)\right]}{[1/(T-2n-2)] \times \left(\sum u_1^2 + \sum u_2^2\right)} \tag{8}$$

where T is the total number of observations, n is the number of independent variables included into the regression equation, $m = n + 1$, Σu_0^2 is the residual of the joined data set, Σu_1^2 and Σu_2^2 are the residuals of the two subsets of the data.

Accumulation of flufenamic acid in the stratum corneum after penetration from semisolid triglycerides and petrolatum + eutanol illustrated by Fig. 3 is represented for example:

1. The common regression calculation with the observations of both these test series (Table 1) resulted in the following equations:

$$m_{flu} = 347.6 - 12.72 \times m_{ster} \quad (\text{incl./excl. criteria } \leq 0.05/\geq 0.07) \tag{9a}$$

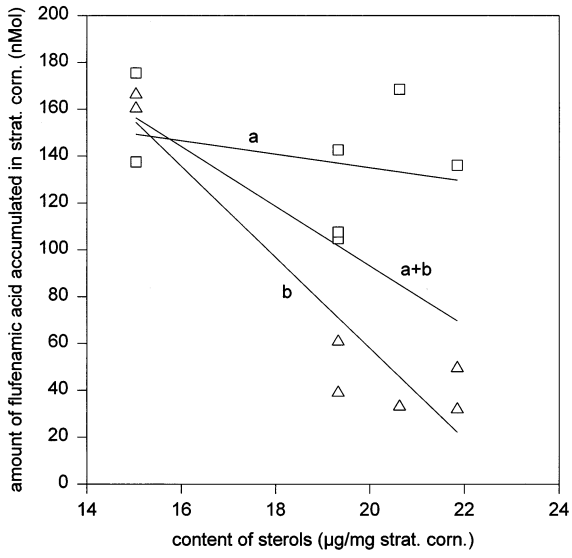

Fig. 3. Chow's test of structural change: linear regression of flufenamic acid accumulation in the stratum corneum versus horny layer content of sterols after penetration from semisolid triglycerides (□ = a) and petrolatum + eutanol (△ = b). a + b is the common regression of both the data sets resulting in inclusion of sterols as the independent variable.

residual $\Sigma u_0^2 = 24059$

$$m_{flu} = 343.3 - 15.05 \times m_{ster} + 2.79 \times m_{ffa}$$

(incl./excl. criteria $\leq 0.50/\geq 0.52$) (9b)

residual $\Sigma\, u_0^2 = 22923$

2. Eq. (9a) has been used for generating Fig. 3 because of its simpler and, therefore, more instructive graphic. Accordingly, the content of sterols as independent variable has been inserted into the regression equations separately calculated with the subsets of both the preparations yielding Eq. (10) and Eq. (11).

(a) Penetration from semisolid triglycerides:

$$m_{flu} = 192.4 - 2.87 \times m_{ster} \quad (10)$$

residual $\Sigma\, u_1^2 = 4027$

(b) Penetration from petrolatum + eutanol:

$$m_{flu} = 446.2 - 19.41 \times m_{ster} \quad (11)$$

residual $\Sigma\, u_2^2 = 2317$

3. Comparison of the F-values calculated by Eq. (8) (= 13.96) and tabulated as percentiles of F distribution (= 7.56, level of significance = 99%) verifies the difference between the regression lines a and b which is evident from Fig. 3 also. Calculation using Eq. (9b) results in a little higher F_{cal} (= 14.20) which is to compare with F_{tab} (= 7.59) showing a corresponding level of significance.

The results of significance tests for structural change are summarized in Table 4c. It is evident that the discrimination between the effects of different vehicles is more sensitive with Chow's test than with the two-independent-groups t-test of the means. This is based on compensating systematic influences of lipid components on the observations by linear regression. Thereby, the significance test is improved by reducing the error according to:

sum of squares about regression (residual) = sum of squares about the mean − sum of squares due to regression (Draper and Smith, 1981).

Comparing the results of the paired-sample t-test and the test of structural change (Table 4b and c) shows 14 cases (25 all in all) which are significantly differentiated at the same level. Significant differences at a higher level compared to the paired t-test are detected with Chow's test for eight pairs. Looking at the regression coefficients elucidates that high significance levels are attained, if the regression equations have high correlation coefficients indicating the degree of compensation for the influence of lipid components. The last-mentioned eight pairs accordingly result in equations with relatively high r-values, whereas the three cases having lower significance levels than with the paired-sample test show low correlation coefficients. These are some observations of hydrocortisone accumulated in the dermis in low quantities of which four measurements only were obtained because of lack of skin material. On the other hand, it is an advantage of this method that even sample groups with large ranges can be differentiated from one another, if the regression equation shows high correlation: Flufenamic acid incorporated into petrolatum + eutanol (S.D. of accumulated drug quantity = ± 77%) is such an example which

Table 4
Differentiation of vehicle effects on drug accumulation in stratum corneum and dermis of excised human skin[a]

	Stratum corneum				Dermis			
	Significance levels of the differences between the formulations tested (%)							
Drug	99	95	90	Insign. (<90)	99	95	90	Insign. (<90)
(a) Two-independent-groups t-test								
Flu	pet<stri, pet<estr	epet<stri	epet<estr	pet/epet, stri/estr				pet/epet, pet/stri, pet/estr, stri/epet, stri/estr, epet/estr
Eu	epet<estr							
Hc				pet/wac, pet/stri, pet/nhc, wac/stri, wac/nhc, stri/nhc		pet<nhc, wac<nhc	wac<stri	pet/wac, pet/stri, stri/nhc
(b) Paired sample t-test								
Flu	pet<stri, pet<estr		epet<stri, epet<estr	pet/epet, stri/estr		pet<stri, epet<stri	epet<estr	pet/epet, pet/estr, stri/estr
Eu		epet<estr						
Hc	wac<pet	wac<stri, wac<nhc		pet/stri, pet/nhc, stri/nhc		wac<nhc, wac<stri	pet<stri	pet/wac, pet/nhc, stri/nhc
(c) Multiple linear regression followed by F-test of structural change								
Flu	pet<epet, pet<stri, pet<estr, epet<stri, epet<estr			stri/estr	pet<estr, epet<stri, epet<estr, stri<estr	pet<stri		pet/epet
Eu	epet<estr							
Hc	wac<pet	wac<stri, wac<nhc		pet/stri, pet/nhc, stri/nhc			wac<nhc	pet/wac, pet/stri, pet/nhc, wac/stri, stri/nhc

[a] (a) Comparisons of means from two independent groups of observations with unequal sizes and variances by *t*-test. (b) Comparisons of means by paired sample *t*-test. (c) Multiple linear regression followed by Chow's *F*-test of structural change. Flu, flufenamic acid; hc, hydrocortisone; eu, eutanol®. Vehicles: pet, white petrolatum; epet, white petrolatum+eutanol® (9:1); stri, semisolid triglycerides; estr, semisolid triglycerides+eutanol® (9:1); nhc, nonionic hydrophilic cream; wac, wool alcohols cream. Insign., insignificant.

yields differences at the highest level of significance by means of Chow's test. Further improvement of this method may be within reach by quantifying the accumulated drug and the contents of lipid classes in the same piece of the horny layer so that these values belong to an identical area.

To some biopharmaceutical points of view, e.g. pharmacokinetics, direct comparisons of accumulation values may be informative and useful. The observations and their means, however, suffer from the dependence on the lipid composition as discussed above. The best region for comparing, therefore, may be the smallest range of the confidence intervals of the regression curves which is situated at the arithmetic means of the independent variables (content of the respective lipid class $= x_i$). Eq. (12a) gives the confidence limits y' for every x value (Bolton, 1997):

$$y' = y_i \pm t \times s_{y,x} \times \left[1/N + \frac{(x_i - x_{am})^2}{\sum (x_i - x_{am})^2} \right]^{0.5} \quad (12a)$$

where y_i is the drug quantity calculated with the regression equation, t is the t-distribution value in dependence on degree of freedom and probability, $s_{y,x}$ is the S.E. of estimate given by regression calculation, N is the number of observations, x_{am} is the arithmetic mean of the contents x_i of the lipid class.

If $x_i = x_{am}$, the term $x_i - x_{am}$ and the fraction will be zero, then, Eq. (12a) becomes:

$$y' = y_i \pm t \times s_{y,x} \times (1/N)^{0.5} \quad (12b)$$

Eq. (12b) is also valid for confidence intervals of multiple linear equations, as all the fractions related to the respective means of the lipid classes will be zero. It is suggested, therefore, to calculate an 'avarage value of accumulation' (ava) which is related to the respective mean contents of the lipid components x_{am}. The S.E. of estimate, $s_{y,x}$, needed for the calculation of the confidence limits of ava and obtained from the regression calculation is lower than the S.D. of the arithmetic mean of the observations (see above). The confidence intervals of ava, therefore, are smaller than those of the arithmetic means. For the comparison of ava values, however, it is likewise required that the independent variables of the regression equations used for the calculation of ava are identical. Hence, the data of the tests which are to be compared were subjected to a common regression as described above. In consequence, there is no advantage over the F-test for structural change but over the t-test with the arithmetic means when verifying significant differences.

Drug accumulation in the horny layer is an important parameter which local drug effects and transdermal drug fluxes depend on. The enrichment of flufenamic acid, for example, decreases in the significantly established sequence of vehicles used for application: semisolid triglycerides $\approx$ semisolid triglycerides + eutanol > petrolatum + eutanol > petrolatum. In this manner effects of vehicle components can be discovered: Eutanol combined with petrolatum raises the accumulation in comparison to the last-mentioned base. In combination with semisolid triglycerides, however, it cannot enhance the enrichment over the effect of the triglycerides. The permeation through the stratum corneum may be considered by drug accumulation in the dermis using the penetration method presented. The drug quantities enriched in this tissue can be correlated also with the lipid composition of the horny layer, and significant differences are to be verified by the F-test for structural change. A summary of the results obtained with flufenamic acid and hydrocortisone is given in Table 4c.

4. Conclusions

The lipid composition of the stratum corneum systematically influences solubility and permeability of drug substances thereby causing high variability of accumulation and permeation measurements. Such systematic influences can be compensated for by (multiple) linear regression, and Chow's F-test for structural change is suitable for detecting sensitively significant differences between test series. It should not be overlooked, however, that statistical methods need a sufficient number of data. It is desirable, therefore, that this method applied to biopharmaceutical problems

for the first time will be supported and confirmed by further measurements. Additional examples may show how far this procedure is suited to other areas of investigation, e.g. barrier transport using other biological materials.

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