ELSEVIER

International Journal of Pharmaceutics 111 (1994) 7–14

international journal of pharmaceutics

Percutaneous penetration in vivo of amino acids

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Received 16 February 1994; accepted 11 March 1994

Abstract

An alternative analytical methodology has been developed to evaluate the in vivo percutaneous penetration of non-radioactive compounds in human stratum corneum by the stripping technique. Different amino acids were applied and even though a slight influence of their structure was found on percutaneous penetration, some topical application strategies such as vehicles, skin preparation and evaporation conditions were considered to be important for skin permeation. The amino acid amount found in the stratum corneum could probably be sufficient to express its moisturizing action.

Key words: Percutaneous penetration; Permeability; Amino acid; Stripping; Human skin

1. Introduction

In the last few decades, special attention has been paid to our understanding of the mechanisms involved in the penetration of many chemicals through the skin. Regardless of the different mechanisms implicated in percutaneous absorption, the stratum corneum plays an important role as a permeation barrier and as a possible reservoir of the molecules topically applied.

Rougier et al. (1987, 1988) have published many papers on the stripping technique in vivo, which could be very useful in studying percutaneous absorption. For a wide series of chemical compounds, a linear relationship between the amount of a certain compound present in the stratum corneum after 30 min of topical application time and the amount that penetrated at a systemic level for a period of 4 days was established. This relationship is not dependent on several factors such as application time, dose, vehicle, anatomic site, etc. (Rougier et al., 1983; Rougier and Lotte, 1987).

The total absorption of a given compound can be predicted by sequential strippings of the skin site treated, determining the amount of the compound present in the stratum corneum after 30 min of its topical application.

The results published on this technique are useful for studying the percutaneous absorption of different compounds as well as the influence of

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SSDI 0378-5173(94)00087-L

some vehicles in both animals and human beings. However, the stripping technique is based on the use of radiolabelled compounds given the higher analytical sensitivity. This approach is controversial when taking into account possible application to humans.

In recent years, some papers have been published on the use of non-radioactive assays to evaluate the in vivo penetration of compounds such as erythromycin or lanolin in the upper skin layers in man (Clark, 1992; Van Hoogdalem, 1992). Following this trend, in the present work an analytical methodology based on stripping of human stratum corneum has also been developed with non-radioactive compounds, using different amino acids, which can be analytically quantified with conventional but specific techniques such as the use of an amino acid autoanalyzer or HPLC.

Amino acids are known to be a component of the skin natural moisturizing factor (NMF) which serves as a sponge keeping water in the stratum corneum and imparting a subjective sensation of smooth skin. For this reason, amino acids have been widely applied in the cosmetic industry. Subsequent studies demonstrated that the presence of amino acids in the stratum corneum caused an increase in the penetration of aqueous solution into the lipids. Their presence in a lipid model, considered by Friberg and Ma (1993), made its interlayer spacing less dependent on the water content and had a significant effect on the water holding capacity of lipids.

Surprisingly, studies on skin penetration of amino acids in cosmetics have not yet been carried out. Despite the commonly held opinion that the amino acids penetrate the skin, most scientists who work in this field of percutaneous absorption have reasons to suspect that such penetration may not occur at all (Sznitowska, 1993). In the literature, there are only data about in vitro studies on their penetration through hairless mouse skin from and only one reference to systematic studies on percutaneous absorption of amino acids (Ruland and Kreuter, 1991).

Hence, the main purpose of this investigation was to develop an alternative analytical methodology to evaluate in vivo percutaneous penetration of different free amino acids.

2. Materials and methods

The amino acids used in this work were purchased from Merck and Fluka. They were all reagent grade and were used without further purification. The solvents used, especially ethyl acetate (Merck) and methanol (Fluka), were of analytical grade quality.

Sequential stripping of the stratum corneum was carried out by using Scotch Magic 3M adhesive tape. In the analytical preparation of the amino acid samples, Millipore filters (GS WP O2500) and Ultrafree filtration units (MC UFC3 LGC00) were used.

The amino acid content of the different samples extracted from the corresponding strippings was determined by an Amino Acid Autoanalyzer (Biotronic LC5001) based on the use of an ionic interchange separation column. Additionally, an HPLC technique (Waters), specifically prepared for the detection of amino acids from their phenylthiocarbamylamino acid derivatives was used in order to obtain another analytical assessment for amino acid estimation. The detection limits for each technique were: 0.10 and 0.005–0.01 nM, respectively.

Topical application assays and stratum corneum strippings were performed on a human volunteer in a conditioned room at 22°C with 60% relative humidity. The test subject had been previously permitted to become acclimatised for 30 min under these conditions.

Application areas of 4 cm^2 were delimited with an adhesive cell at the cubital surface on either the right or left forearm of the subject.

Solutions containing the different amino acids assayed were applied (15 μl) in the delimited area previously cleaned with a Kleenex. These solutions led in all cases to an amino acid amount of 2000 nmol in the total application surface (500 nmol cm^{-2}).

After 30 min of topical application, tape stripping was commenced with Scotch Magic 3M adhesive tape. In the first stripping the amino acid content analyzed was that which slightly entered the stratum corneum or did not penetrate at all. The next 15 strippings, collected in groups of five, allowed us to determine the amino acid content

which penetrates the different layers of the stratum corneum.

Each tape (first tape) or group of tapes (2nd to 6th, 7th to 11th and 12th to 16th) were collected into centrifuge tubes and 20 μl of an internal standard of norleucine solution (40 nM/μl) were added. Ethyl acetate (5 ml) was added and after complete solubilization of the adhesive tape (60 min), selective precipitation of the adhesive polymer was achieved by adding 20 ml of methanol. After 10 min centrifugation at 5000 rpm, 15 ml of the upper phase was evaporated to dryness on a rotary evaporator (Univap GV2) at 2800 rpm and 45°C for 2 h. The dry residue was dissolved in 2 ml of pH 2.2 citrate buffer to be determined by an amino acid autoanalyzer. Specific filtration with Ultrafree-MC UFC3 LGC00 (Millipore) was performed to separate the free amino acids from the keratinic protein of the stratum corneum which could have been stripped and extracted by this procedure. Therefore, a 2 ml solution was introduced in specific cylinders provided by a filter membrane and, after 30 min at 7500 rpm in an Eppendorf centrifuge (S415), the filtered solutions were stored at 4°C until analysis.

3. Results and discussion

Previous analyses on free amino acids present in the stratum corneum were performed in order to choose accurately the most appropriate amino acids to be applied to the skin. The stripping and extraction procedures were performed using norleucine as an internal standard since this amino acid is not found in a conventional proteinic structure. Free amino acid concentrations in the different stripping groups of the stratum corneum are listed in Table 1. It should be pointed out that to obtain the free amino acids in the different stratum corneum layers, hydrolysis was not performed and proteins and peptides were removed by filtration. An additional column has been included in Table 1 on the amino acid composition (percentage) of hydrolized skin keratin (Goldsmith, 1984).

Bearing in mind that quantification of amino acids is representative only for values higher than 0.10 nM/50 μl, we should point out the small amount of ionized amino acids in the stratum corneum (absence of acidic amino acids and only 11% histidine as a basic amino acid), the pres-

Table 1
Free amino acid content in the different strippings of the stratum corneum

	1st stripping	2nd–6th stripping		7th–11th stripping		12th–16th stripping		Amino acid composition of skin keratin (%)
		nM/50 μl	%	nM/50 μl	%	nM/50 μl	%	
Aspartic acid	ND	ND		ND		ND		7.7
Threonine	ND	0.50	7.9	0.55	7.6	0.42	7.4	3.1
Serine	0.14	2.48	39.2	2.85	39.6	2.22	38.9	12.4
Glutamic acid	ND	ND		ND		ND		14.3
Proline	ND	ND		ND		ND		1.1
Glycine	ND	1.30	20.6	1.51	21.0	1.22	21.4	26.9
Alanine	ND	1.11	17.6	1.27	17.6	0.98	17.2	4.2
Valine	ND	0.22	3.5	0.23	3.2	0.20	3.5	2.5
Methionine	ND	ND		ND		ND		1.8
Isoleucine	ND	ND		ND		ND		2.1
Leucine	ND	ND		ND		ND		7.0
Tyrosine	ND	ND		ND		ND		2.72
Phenylalanine	ND	ND		ND		ND		2.7
Histidine	0.44	0.71	11.2	0.79	11.0	0.67	11.7	1.2
Lysine	ND	ND		ND		ND		3.6
Arginine	ND	ND		ND		ND		3.9

ence of around 42% apolar amino acids (21% glycine, 17.5% alanine and 3.5% valine) and the high amount of polar amino acids (47%), serine being the free amino acid found in the highest proportion (about 39%). Comparing these values with the amino acid percentages of keratin proteins of skin, it can be deduced that the amino acids present in free state do not correspond with the amino acid existing in higher proportions considering the macrostructure of keratin proteins. On the other hand, Schwarz (1974) reported quantitative free amino acid information in the stratum corneum. The highest proportion of serine followed by glycine, alanine and histidine, should also be noted.

From these results, the following amino acids were considered to be the most suitable in order to avoid the small amount of free amino acids present in the skin: glutamic acid, isoleucine, lysine and threonine. As can be seen in Table 1, no glutamic acid, isoleucine and lysine were detected as free amino acids. Since threonine is the only amino acid detected (about 0.5 nM/50 μl), this amount was subtracted from the total content analyzed after the stripping and extraction procedure (the total amount analyzed was about 30 nM/50 μl). Owing to some difficulties in solubilization for glutamic acid with respect to the other amino acids, most assays of percutaneous penetration were performed with threonine, isoleucine and lysine using water and MeOH/H_2O (20:80) as vehicles.

The study on percutaneous absorption of amino acids was focused, in a first step, on the determination of the influence of different characteristics of the amino acids used in the experiments. Taking into account the isoelectric points of threonine (6.2), isoleucine (6.0) and lysine (9.8), the pH of the aqueous solutions (about 6.1) of amino acids and the accepted pH of the skin (about 5.5), during the permeation through the stratum corneum, threonine and isoleucine can be regarded as unionized or slightly electropositive species and lysine as clearly electropositive in character. According to a molecular structure criterion, threonine can be considered to be a polar amino acid and isoleucine a non-polar one.

The amino acid solutions were topically applied, the time of dryness being 20–25 min. Tape stripping of the stratum corneum commenced 30 min after the application. After the extraction of amino acids from the different tapes, the samples

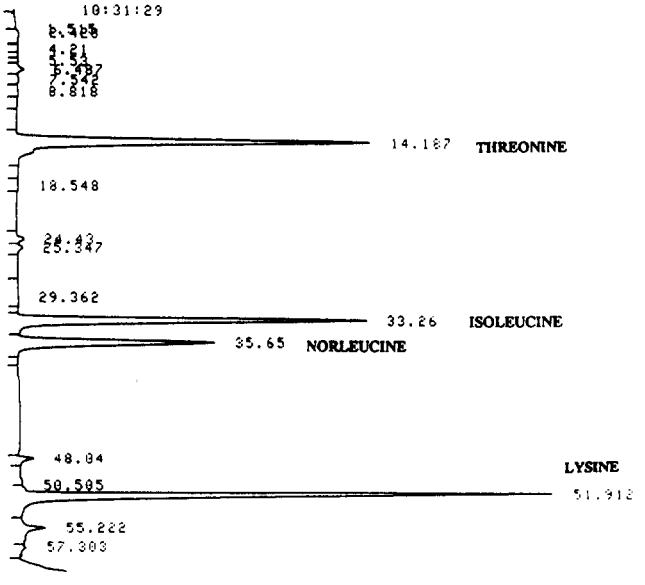

Fig. 1. Representative analytical chromatogram obtained by an amino acid autoanalyzer in the determination of the amino acids chosen in this work.

Table 2
Percentage values of the amino acids present in the different strippings after topical application of an aqueous solution of amino acids

	1st stripping %	2nd–6th stripping %	7th–11th stripping %	12th–16th stripping %	Total %	stratum corneum % (but 1st strip)
Threonine	57.6	25.1	1.9	0.8	85.4	27.8
Isoleucine	65.2	27.4	2.6	0.9	96.1	30.9
Lysine	56.8	26.7	2.3	0.7	86.5	29.7

were analyzed. In Fig. 1 a representative pattern of these compounds is shown and, as can be seen, both good identification and detection including norleucine as the internal standard were achieved. The similar values obtained using the amino acid autoanalyzer and HPLC techniques confirm the validity of both analytical methodologies.

Mean results of duplicate experiments on percutaneous absorption of amino acids from aqueous solutions obtained with the amino acid autoanalyzer are listed in Table 2. The amino acid content has been expressed as a percentage of the total amount applied on the skin surface. In all cases, the values of the internal standard (norleucine), the analysis of the reference samples and the amount of free amino acids present in the different stripping (blank assay) were taken into consideretion to obtain the final corrected values.

From the results indicated in Table 2, it can be deduced that the total amount analyzed was greater than 85%, isoleucine being the amino acid found in the highest amount. The amino acid content in the first strip, which accounts for the non-penetrating content, is the most important, decreasing drastically in the next stripping groups. A similar tendency in percutaneous penetration could be observed for all the amino acids studied. It should be noted that isoleucine is the amino acid present in the highest amount both in the external layer of the stratum corneum (65% 1st strip) and also in the internal layers of the stratum corneum (30% SC).

This implies that the stratum corneum has a

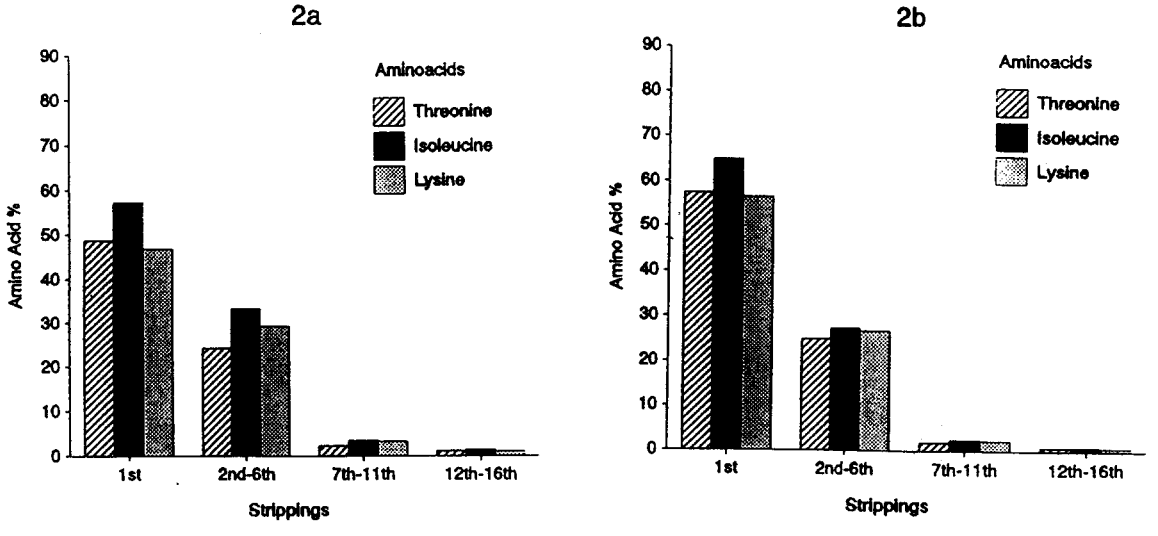

Fig. 2. Variation of the amino acid percentages as a function of the strippings of the stratum corneum. (a) Methanol/H_2O (20:80). (b) Aqueous solution.

greater barrier effect for isoleucine than for lysine and threonine which could easily penetrate the inner layers. The similar values obtained for lysine and threonine indicate that the electropositive charge of lysine does not seem to be an important factor during its percutaneous penetration.

The application vehicle was changed in order to study its influence on the percutaneous absorption of the amino acids chosen. In Fig. 2a the results obtained after topical application of threonine, isoleucine and lysine in a methanol/H_2O (20:80) vehicle are shown. In order to compare these results with the percentage values on percutaneous absorption obtained for those amino acids in aqueous solutions, the data of Table 2 are graphically plotted in Fig. 2b. It can be deduced in general that an aqueous-methanolic solution enhances the diffusion into the stratum corneum of the amino acid studied. As can be seen, the amino acid content in the first stripping is lower in MeOH/H_2O than in aqueous solution. However, the amounts of amino acid in the different layers of the stratum corneum are similar to or even slightly higher in the case of isoleucine and lysine.

It seems that the MeOH/H_2O (20:80) vehicle enhances the initial penetration of the amino acids, favouring their subsequent distribution through the inner layers of the stratum corneum in the case of isoleucine and in the more internal fractions for threonine and lysine.

A key factor implicated in the percutaneous penetration of amino acids could be the initial step in the penetration through the skin surface. To study this phenomenon two different set of experiments were also carried out.

In the first assay, the drying of aqueous solutions of amino acids on the skin was enhanced by applying air ventilation. Under these conditions, dryness was achieved after 10–15 min instead of the 20–25 min under normalized ambient conditions. The stripping of the stratum corneum was also started, after 30 min of topical application of the amino acid solution. The detection of the different amino acids in each stripping group is illustrated in Fig. 3a.

It can be shown that, using forced drying conditions, the amount of amino acids present in the first stripping increases markedly and the amino acid content in the other stripping groups clearly decreases. However, as occurred in the previous assays, there is also a greater amount of isoleucine in the external and the internal stratum corneum layers when compared with threonine and lysine. Possibly, a diminution of the vehicle action of the

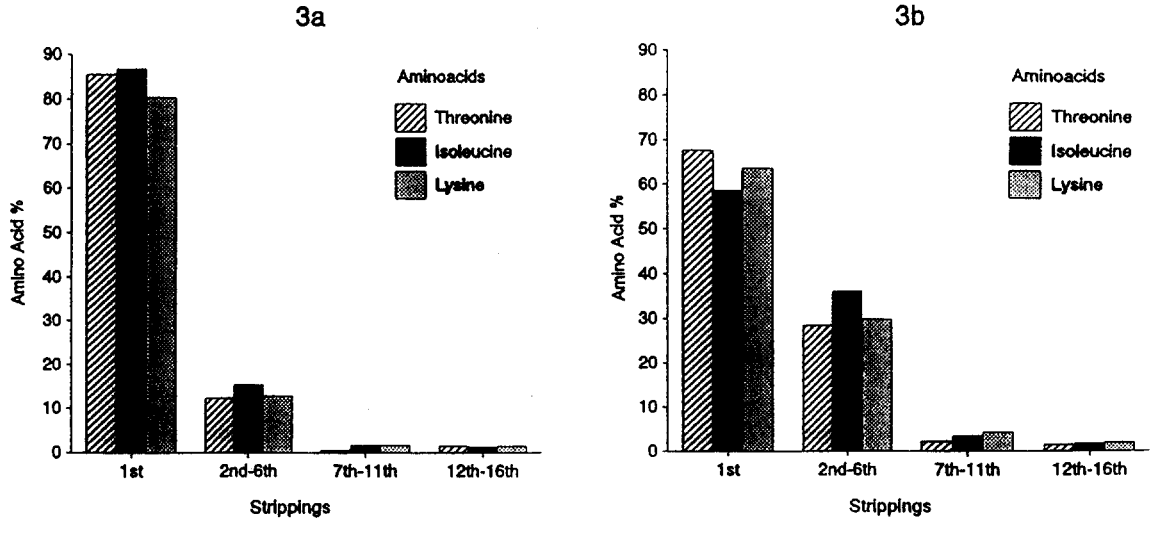

Fig. 3. Amino acid percentages for each stripping group. (a) Forced skin drying; (b) previous single skin stripping before application of an amino acid aqueous solution.

solvent due to forced dryness and a modification of the normal mechanisms of transepidermal evaporation of the skin can induce a certain inhibition of percutaneous penetration to a similar extent for all amino acids assayed.

A second assay based on a previous single stripping of the skin before the topical application of an amino acid solution was carried out. Under these conditions the dryness time was slightly longer (25–30 min) than under conventional conditions. Immediately after the total drying of the skin, stripping of the stratum corneum was performed and the analytical results obtained on the amino acid for each stripping group are plotted in Fig. 3b.

From these results, it can be appreciated that in the stripping groups corresponding to the inner layers of the stratum corneum, higher amounts of amino acids are detected with respect to those obtained in aqueous solutions under normal conditions (Fig. 2b). This is even more remarkable with respect to the amounts obtained under forced drying conditions (Fig. 3a). The lower amount of isoleucine in the external layer of the stratum corneum could imply that a previous single strip influences mainly the percutaneous barrier effect of the apolar amino acid isoleucine.

The values obtained for percutaneous penetration in the stratum corneum of the different amino acids under the different experimental conditions are summarized in Table 3.

In all cases a higher proportion of isoleucine was found in the stratum corneum, followed by lysine and threonine. Nevertheless, a clear distinction between the different chemical characteristics of amino acids (charged or uncharged, polar or non-polar) and their permeation through the skin was not found. Recent studies (Ruland and Kreuter, 1991, 1992) indicated that amino acids cannot penetrate into and permeate through full-thickness hairless mouse skin in significant amounts after dermal application. A significant difference between the permeation of positively and negatively charged amino acids was not obvious; likewise, hairless mouse skin did not form a reservoir for amino acids. Even with the use of penetration enhancers, the total content of amino acids in the skin was only 0.4% of the initial amount applied. However, it should be borne in mind that in vitro permeation through hairless mouse skin is not necessarily representative of permeation in human skin.

In our case, the absolute amount of amino acids ranged from 15 to 25 $\mu g\ cm^{-2}$ for the two initial experiments in which the skin surface was not modified. Published data concerning this subject are equivocal. Ruland and Kreuter (1991) did not find significant deposition of amino acids in the skin, whereas Wearley et al. (1990) concluded that binding of amino acids in stratum corneum does occur. Sznitowska (1993) reported that, from in vitro studies with dermatomed human cadaver skin, the concentration of amino acids measured in stratum corneum ranged from 1.5 to 6.3 $\mu g\ cm^{-2}$, despite the lack of evidence of skin binding.

The amount of amino acids penetrated into the stratum corneum can be important for cosmetological purposes; in particular, an amino acid concentration range of 15–25 $\mu g\ cm^{-2}$ could be interesting to be considered in order to obtain a certain moisturizing action. Possibly, the amino acid penetration level could be increased using more suitable vehicle strategies.

Table 3
Amino acid amount (% and $\mu g\ cm^{-2}$) in the stratum corneum obtained when applied in water (W), in methanol/water 20:80 (M/W), in water with forced skin drying (W/D) and in water in a previous stripped skin surface (W/S)

	W		M/W		W/D		W/S	
	%	$\mu g\ cm^{-2}$	%	$\mu g\ cm^{-2}$	%	$\mu g\ cm^{-2}$	%	$\mu g\ cm^{-2}$
Threonine	27.8	16.5	27.7	16.5	14.3	8.5	32.1	19.1
Isoleucine	30.9	20.2	38.3	25.1	18.3	12.0	41.3	27.0
Lysine	29.7	21.7	33.7	24.6	16.0	11.7	36.0	26.3

4. Conclusions

A new non-radioactive method to evaluate the in vivo percutaneous penetration of compounds into human stratum corneum by the stripping technique has been designed based on the use of amino acids analytically quantified with conventional techniques such as the use of an amino acid autoanalyzer or HPLC.

From the experiments presented here, suitable amounts of amino acids in the different stripping groups have been found enabling us to follow the transdermal permeability of each amino acid through the inner layers of the stratum corneum.

Little influence of the amino acid chemical structure on percutaneous penetration was found. A greater barrier effect tendency was observed only for isoleucine, the non-polar amino acid, in contrast to the polar amino acid (threonine) and the electropositive charged amino acid (lysine).

The importance of some topical application strategies for amino acid percutaneous penetration has been studied including the vehicle, the skin preparation and also the evaporation conditions. These are key factors to be considered while evaluating skin permeation of these compounds.

Even though amino acids have no affinity with the lipids of the intercellular domains of stratum corneum, a certain amount was found to be absorbed, from 15 to 25 $\mu g\ cm^{-2}$, which could probably be sufficient to express their moisturizing action.

Acknowledgements

The authors are indebted to Col.legi Oficial de Farmacèutics from Barcelona (Spain) for financial support of this project. We are also grateful to Drs D. Caron, J.C. Caron and Professor H. Schaefer from Centre International de Recherches Dermatologiques CIRD-Galderma (France) for all their kindness and dedication in teaching one of us (M.O.) the stripping technique methodology. Thanks are also due to Mr J. Caelles for valuable assistance in the amino acid determinations.

References

Clark, E.W., Short-term penetration of lanolin into human stratum corneum. *J. Soc. Cosmet. Chem.*, 43 (1992) 219–227.

Friberg, S.E. and Ma, Z., Partition of free amino acids between stratum corneum model lipids and corneocytes. *J. Dispersion Sci. Technol.*, 14 (1993) 481–498.

Goldsmith, M.O., *Biochemistry and Physiology of the Skin*, Oxford University Press, Oxford, 1984, Chap. 4, pp. 135–169.

Rougier, A. and Lotte, C., Correlations between horny layer concentration and percutaneous absorption. *Pharmacol. Skin*, 1, (1987) 81–102.

Rougier, A., Dupuis, O., Lotte, C., Roguet, R. and Schaefer, H., In vivo correlation between stratum corneum reservoir function and percutaneous absorption. *J. Invest. Dermatol.*, 81 (1983) 275–278.

Rougier, A., Lotte, C., Corcuff, P. and Maibach, H.I., Relationship between skin permeability and corneocyte size according to anatomic site, age and sex in man. *J. Soc. Cosmet. Chem.*, 39 (1988) 15–26.

Rougier, A., Lotte, C. and Dupuis, D., An original predictive method for in vivo percutaneous absorption studies. *J. Soc. Cosmet. Chem.*, 38 (1987) 397–417.

Ruland, A. and Kreuter, J., Transdermal permeability and skin accumulation of amino acids. *Int. J. Pharm.*, 72 (1991) 149–155.

Ruland, A. and Kreuter, J., Influence of various penetration enhancers on the in vitro permeation of amino acids across hairless mouse skin. *Int. J. Pharm.*, 85 (1992) 7–17.

Schwarz, E., Biochemische Stigmata menschlicher Hautoberfläche im Alter. *Z. Klin. Chem. Klin. Biochem.*, 12, (1974) 93–97.

Sznitowska, M., Percutaneous absorption of amino acids and their cosmetic efficacy. *Cosmet. Toiletries*, 108, (1993) 29–32.

Van Hoogdalem, E.Y., Assay of erythromycin in tape strips of human stratum corneum and some preliminary results in man. *Skin Pharmacol.*, 5 (1992) 124–128.

Wearley, L.L., Tojo, K. and Chien Y.W. A numerical approach to study the effect of binding on the iontophoretic transport of a series of amino acids. *J. Pharm. Sci.*, 79 (1990) 992–998.