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Research paper

Comparative permeation studies for δ-aminolevulinic acid and its *n*-butyl ester through stratum corneum and artificial skin constructs

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Abstract

The improvement of the permeation properties through excised human stratum corneum and artificial skin constructs (ASC) of δ-aminolevulinic-*n*-butylester (ABE) compared with δ-aminolevulinic acid (ALA) was investigated. For this purpose the permeated amounts of each substance were determined depending on time in a Franz diffusion cell experiment with stratum corneum and ASC, respectively. Furthermore the barrier properties of ASC were compared with those of stratum corneum. Detection of both substances was performed by high-performance liquid chromatography (HPLC) analysis. For the determination of ABE a new HPLC method was developed. ABE could be determined with the new HPLC method with sufficient sensitivity (detection limit: 0.1 μg/ml) after derivatisation with *o*-phthalaldehyde (OPA). Stratum corneum and ASC were more permeable for ABE than for ALA. The permeation coefficient *P* of ABE through stratum corneum was nearly ten-fold higher than that of ALA. Using ASC as permeation barrier the permeation coefficient of ABE was about 22-fold higher than that of ALA. ABE and ALA permeated 142-fold and 64-fold, respectively, faster through ASC than through stratum corneum.

Keywords: δ-Aminolevulinic acid; Analysis of δ-aminolevulinic acid-*n*-butyl ester; ALA derivatives; Artificial skin constructs; Stratum corneum; Permeation studies

1. Introduction

δ-Aminolevulinic acid (ALA) acts as a precursor in the biosynthetic pathway of porphyrins, especially protoporphyrin IX (Pp IX), and heme in human body [1]. ALA is applied topically as a prodrug of Pp IX and thus sensitising in photodynamic therapy (PDT) to treat special skin tumours or cancer of the bladder, oesophagus, and lung [2,3]. PDT uses the increase in Pp IX concentration in abnormal cells after topical or systemic application of exogenous ALA. After accumulation of Pp IX within the malignant tissue the fluorescence of Pp IX is activated by light of an appropriate wavelength to destroy the malignant tissue [4–8]. Topical application of ALA is usually performed with water in oil emulsions, enriched with ALA in concentrations between 10 and 20% [3,6,9,10].

A major drawback of ALA is its hydrophilicity as a hydrochloride. Hence, ALA shows only limited permeation through stratum corneum, the lipophilic barrier of the skin. To improve dermal bioavailability of ALA, a variety of ALA esters were synthesised [11–15], of which ABE induced higher Pp IX concentration in cultivated cells. Uehlinger et al. [16] determined the apparent partition coefficients of ALA and its esters (octanol-buffer system at 21°C and pH 7.4). They indicated an increase in lipophilicity of these esters.

Another disadvantage of ALA is a well investigated instability in aqueous solution at a pH above 5.2. Destabilisation increases with increasing pH. This instability is recognized by an increase in yellow colour and a decrease in pH of the solution due to a formation of pyrazin derivatives [17–19]. At low temperature and a pH of 5.0, which is similar to the pH of stratum corneum yet undergoing interindividual variations [20–22], stability can be guaranteed for several days [19].

Expecting low permeation of ALA and ABE in the in vitro permeation studies a very sensitive quantification of both substances is required. Because of an insufficient UV detection sensitivity of ALA a fluorometric detection after derivatisation with *o*-phthalaldehyde (OPA) was used [23–25]. The present study provides a high-performance liquid chromatography (HPLC) method for the quantification of ABE. Derivatisation of ABE was performed according to

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Ref. [23]. Elution of ABE was adopted from Ref. [26] to separate α-amino acids released from stratum corneum or artificial skin construct (ASC), which react the same way.

The disadvantages of using excised human stratum corneum are inter- and intra-individual variations in permeability between different skin samples depending on age, sex, nutrition status, and part of the body where the samples were taken from [27]. ASC can be produced by standardised cultivation [28,29] which is an alternative to excised human skin. Recent permeation studies with ibuprofen formulations have shown [28,29], that excised human stratum corneum can be replaced with ASC. Although barrier function of organotypic cultures of epidermal and dermal cells is less elaborated, differences in permeation for a variety of ibuprofen formulations can be determined in the same rank order with ASC as with excised human stratum corneum.

The aim of the present study was to compare the diffusion properties of ALA and ABE into skin. Since stratum corneum is the main barrier of the skin especially for hydrophilic molecules like ALA, excised human stratum corneum was chosen for the permeation experiments. The barrier properties of stratum corneum for ALA and ABE were compared with those of ASC.

2. Materials and methods

2.1. Materials

ALA hydrochoride was provided by Medac GmbH (Wedel, Germany). ABE hydrochloride was synthesised according to Kloek and Beijersbergen van Henegouwen [11]. *n*-Butanol was purchased from Heraeus GmbH (Karlsruhe, Germany), diethylether (reagent grade), and OPA (HPLC grade) from Fluka (Neu-Ulm, Germany). Thionylchloride (reagent grade), mercaptoethanol (pro analysi), and all buffer substances (pro analysi) were purchased from Merck KGaA (Darmstadt, Germany). Acetonitrile, methanol, and acetic acid (all chemicals were HPLC grade) were purchased from J.T. Baker (VA Deventer, The Netherlands). Boric acid was purchased from Carl Roth GmbH (Karlsruhe, Germany), absolute ethanol (HPLC grade) from Riedel de Haën (Seelze, Germany). Water was used in bidistilled quality.

Excipial® Fettcreme was provided by Hans Karrer GmbH (Königsbrunn, Germany). Isopore® membrane filters (type TMTP, 5.0 μm) were purchased from Millipore (Ireland). Six-well cell culture clusters with Transwell® inserts were purchased from Costar® (Corning, USA).

2.2. Construction of the ASC

ASC was cultivated according to Ref. [28] with a last step modification according to Ref. [29]. Different cell types were combined to simulate the physiological properties of human skin. The dermis equivalent was cultivated with human dermal fibroblasts from new born foreskin specimen according to standard conditions [30]. The dermis was covered with an epidermis equivalent using keratinocytes from stable HaCaT-cell line (*H*uman *a*dult, low *C*alcium, elevated, *T*emperature).

2.3. Preparation of excised human stratum corneum

The used skin samples were taken from the abdominal region of a female donor. Directly after the biopsy the fat of the skin samples was trimmed away. Then the skin samples were frozen by liquid nitrogen and stored at −25°C until separation of stratum corneum by trypsination [31].

2.4. In vitro permeation studies

The in vitro permeation studies were performed with a modified Franz cell [32]. The donor was Excipial® Fettcreme enriched with ALA and ABE (in both cases 10% (w/w)), respectively. The water in oil system Excipial® Fettcreme and drug concentration of 10% were chosen according to prior investigation of other groups, which used lipophilic bases for occlusive conditions enriched with 10–20% ALA [3,6,8–10]. During permeation studies the drug-loaded donor did not show any signs of instability like yellow colour with either ALA or ABE [17–19]. The donor was prepared freshly just before permeation studies. Each drug was mixed for 2.5 min with Excipial® Fettcreme using an Unguator® (GAKO Konietzko GmbH, Bamberg, Germany). Incorporation of ALA and ABE resulted in complete dissolution of either drug in Excipial® Fettcreme which was controlled by light microscopy. Neither ALA nor ABE crystals could be detected in the respective samples. Receiver contained 4.5–6.0 ml of phosphate buffer of pH 5.0 (Ph. Helv. 8) to guarantee for the stability of ALA and ABE. The receiver was maintained at 37°C by a waterbath. The homogenous distribution of permeated drug in the acceptor was guaranteed by a magnetic stirring bar.

Donor and receiver were separated by stratum corneum and ASC, respectively. Stratum corneum was placed on a polycarbonate filter (Isopore® membrane filters, type TMTP, 5.0 μm, Millipore, Ireland) for a higher mechanical stability. ASC was used together with the polycarbonate membrane of the Transwell® insert.

Samples were taken in different intervals. For the permeation studies with stratum corneum, samples were taken up to 35 h as shown in Fig. 2. Furthermore Excipial Fettcreme®/ABE was used in a permeation study lasting for 58 h to investigate the progress of the permeated ABE amounts between 35 and 58 h as shown in Fig. 3. However, stratum corneum originated from a different donor. Using ASC, samples were taken up to 10 h for ABE and 11 h for ALA, respectively, as shown in Fig. 4. The permeated amounts of both substances were measured by HPLC with fluorometric detection after precolumn derivatisation with OPA [23].

2.5. Determination of the solubility of ALA and ABE in the receptor medium

Either ALA or ABE was mixed with phosphate buffer of pH 5.0 (Ph. Helv. 8) in a ratio to maintain a sediment of each substance even after shaking for 2 h at 20°C. The supernatant concentration of ALA and ABE was determined after dilution by HPLC as described in Section 2.6. The concentrations at saturation of ALA and ABE was 63% (w/v) and 64% (w/v), respectively. Therefore sink conditions were maintained during all the permeation experiments.

2.6. HPLC analysis

The analysis was performed with an HPLC system consisting of a fluorescence HPLC monitor RF-353 (Shimadzu, Kyoto, Japan) and a Spectroflow 400 solvent delivery system (Kratos Analytical, NJ, USA) equipped with a Rheodyne 7125 syringe loading sample injector (Rheodyne, Cotati, USA). The injector was fitted with a 20-μl loop. The analytical column (250 mm × 4.6 mm) was connected in line with a guard column (10 mm × 4.6 mm). Both columns were packed with Hypersil ODS (particle size 5 μm, Grom, Herrenberg, Germany).

ALA and ABE were eluted with different mobile phases, because of the higher lipophilicity of ABE. Elution of ALA was performed at ambient temperature with a mixture of an aqueous sodium acetate buffer (22 mM) and methanol in the ratio of 7.0 to 5.0 (ASC: 7.5 to 5.0), adjusted to pH 3.38 with acetic acid according to Ref. [23]. The flow rate was 1.3 ml/min.

An HPLC method for the determination of ABE was developed in relation to Ref. [26] which uses this method for the determination of α-amino acids. An aqueous monobasic potassium phosphate buffer (12.5 mM, pH 7.2) was mixed with acetonitrile in the ratio of 5.5 to 5.0. ABE was isolated by isocratic elution at a flow rate of 1.5 ml/min at ambient temperature. This HPLC method was calibrated repeatedly in the concentration range of 0.5–40 μg/ml. The resulting correlation coefficients r were always higher than 0.999.

Prior to the measurement, derivatisation of ALA and ABE was performed according to Ref. [23]. Aliquots from the receiver enriched with ABE after the permeation studies through ASC were diluted by ratio 1:10 with phosphate buffer of pH 5.0 (Ph. Helv. 8) because of the high concentration of ABE. One hundred microlitres of the derivatisation reagent, a sodium borate buffer of pH 9.5 enriched with OPA and mercaptoethanol, were mixed with 100 μl of the sample. Immediately after a reaction time of exactly 2 min, 100 μl monobasic potassium phosphate buffer (0.1 M) were added to the mixture to stop the reaction. Twenty microlitres of this solution were injected onto the column. The fluorescence of the reaction products was monitored at an excitation wavelength of 330 nm and an emission wavelength of 418 nm.

3. Results and discussion

3.1. Determination of ABE

Several solvent systems with different ratios of acetonitrile and aqueous monobasic potassium phosphate buffer (12.5 mM, pH 7.2) were tested in order to optimise the separation of ABE from other amino acids. Increasing the organic solvent amount resulted in a shorter retention time of ABE, but the separation from other substances decreased. Using the ratios acetonitrile/phosphate buffer (5.5:5.0) and (5.0:5.0) the ABE peak interfered sometimes with other peaks. The detection of ABE succeeded with a ratio of 5.0 to 5.5 at a retention time of about 11 min with an appropriate sensitivity (detection limit: 0.1 μg/ml). Therefore the method proved to be successful for this purpose. A representative chromatogram is shown in Fig. 1. This chromatogram is derived from a sample, which was taken during the in vitro permeation studies through stratum corneum. It shows some peaks beside ABE caused by the amino acids and the derivatisation reagent.

3.2. Diffusion properties of ALA and ABE through stratum corneum

The permeated amount of ALA and ABE through stratum corneum was calculated as quotient of the total amount of ALA or ABE in the receiver and the permeation area of the Franz cell, respectively. Those calculated values (μg/cm^2) were plotted versus time (min) to determine the flux. The resulting permeation profiles are shown in Fig. 2. ALA and ABE show significantly different permeation profiles. The permeated amounts of ABE were significantly higher after 840 min than those of ALA. Permeation data are summarised in Table 1.

Within the first hours of the permeation studies of ABE the ascent of the curve increased. The ascent became linear between 14 and 24 h. The permeation study for 58 h showed

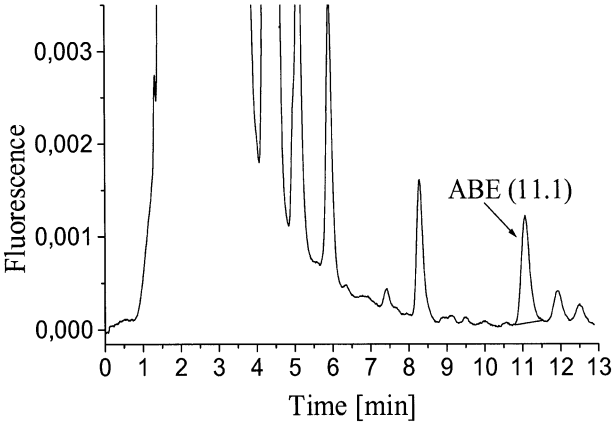

Fig. 1. Chromatogram of the developed HPLC method, concentration of ABE: 1.53 μg/ml.

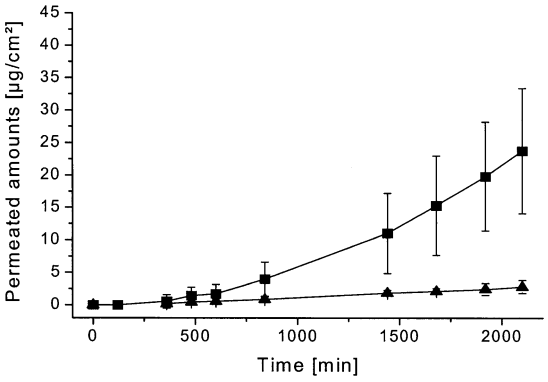

Fig. 2. Permeation from Excipial®-Fettcreme enriched with ALA (10% (w/w)) and ABE (10% (w/w)) across stratum corneum; ■, ALA-*n*-butyl ester, $n = 6$; ▲, ALA, $n = 9$; graphs represent mean values and standard deviation.

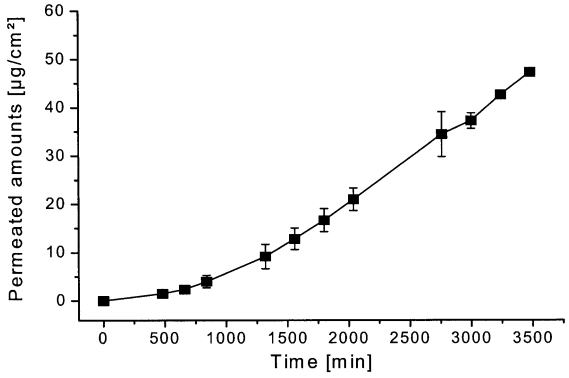

Fig. 3. Permeation from Excipial®-Fettcreme enriched with ABE (10% (w/w)) across stratum corneum for 58 h; graphs represent mean values and standard deviation, $n = 4$.

that linearity of the ascent (correlation coefficient: >0.999) was also found between 35 and 58 h as represented in Fig. 3.

The lag-time for each substance was determined from the intercept of the linear part of the graph with the time axis. For the 35 h permeation studies the calculated lag-time of ABE was 825 min. The permeated amounts of ALA increased linearly after a lag-time of 215 min. For each substance the linear ascent of the curve was used to determine the flux as mentioned above. The permeation coefficient was calculated as quotient of flux and concentration of the drug in Excipial® Fettcreme. Compared with ALA the mean values of flux and permeation coefficient of ABE were nearly ten-fold higher. These results agree with the increase in Pp IX concentration after the dermal application of ALA-esters [12–14]. This increase of the permeation coefficient of ABE through stratum corneum compared with that of ALA, however, does not agree with Uehlinger et al. [16] who estimated that the more lipophilic ABE would penetrate 50 times more efficiently into the skin than ALA. The difference could be explained by different experimental conditions. The results reported in the literature are based on apparent partition coefficients of ALA and ABE, determined at a pH of 7.4 (octanol-buffer system, 21°C). The pH and temperature used in the present in vitro model, were 5.0 and 37°C, respectively, to simulate physiological conditions of the skin and to guarantee for stability of both the substances.

3.3. Diffusion properties of ALA and ABE through ASC

The permeation profiles of ALA and ABE through ASC are shown in Fig. 4. The permeated amounts of ABE increased within the first 3 h of the permeation studies rapidly. After 4–5 h the ascent of the curve became linear. ALA showed the same behaviour. In this case the linear ascent was reached after about 2 h. This rapid increase in permeated amounts agrees with the results of permeation studies through ASC from the literature for other drugs [28]. Each substance may be detected from the beginning of the permeation studies and lag-times are not available. The permeation profiles of both drugs ALA and ABE are significantly different. Again ABE permeated easier than ALA. Permeation data are summarised in Table 2. Flux and permeation coefficient of ABE were about 22 times higher than those of ALA.

3.4. Correlation between stratum corneum and ASC

Permeation studies through stratum corneum as well as through ASC resulted in a faster permeation of ABE than of ALA. ASC was more permeable for the substances than stratum corneum. This agrees with the results of Specht et al. [28]. They compared the permeation coefficients of ibuprofen acid across ASC and stratum corneum using Dolgit-Mikrogel® and Ibutop-Creme® as donors. Ibuprofen acid permeated from both formulations 20 times faster across ASC than across stratum corneum.

For the permeation studies of ALA the permeation coefficient across ASC was 64 times higher compared with the permeation data obtained from the permeation studies through excised human stratum corneum. Comparing the permeation data of ABE the permeation coefficient was

Table 1
Comparison of the permeation data for the permeation studies through stratum corneum

Donor	Flux J (g cm^{-2} s^{-1})	Permeation coefficient P (cm/s)	Lag-time (min)	n
ALA in Excipial®-Fettcreme, 10% (w/w)	2.85×10^{-11} ($\pm 7.04 \times 10^{-12}$)	3.01×10^{-10} ($\pm 7.44 \times 10^{-11}$)	215	9
ABE in Excipial®-Fettcreme, 10% (w/w)	3.19×10^{-10} ($\pm 8.54 \times 10^{-11}$)	3.00×10^{-9} ($\pm 8.04 \times 10^{-10}$)	825	6

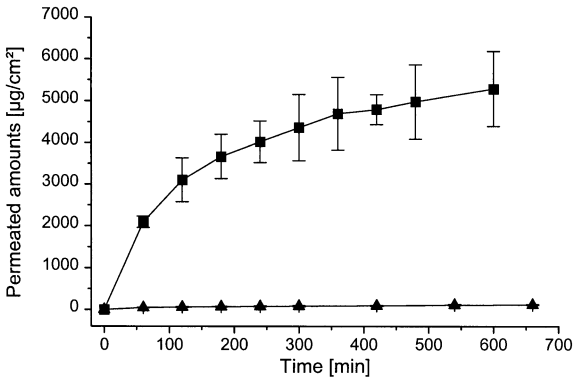

Fig. 4. Permeation from Excipial®-Fettcreme enriched with ALA (10% (w/w)) and ABE (10% (w/w)) across ASC; ■, ALA-*n*-butyl ester, $n = 7$; ▲, ALA, $n = 8$; graphs represent mean values and standard deviation.

142 times higher for ASC than for stratum corneum as represented in Fig. 5. These different factors between the permeation coefficients of the drugs through ASC and stratum corneum could depend on different physicochemical properties of ALA and ABE and on differences in the structure of stratum corneum and ASC. Increase of permeability of ALA and ABE for ASC in comparison with stratum corneum was higher than the increase for ibuprofen acid of the former studies [28,29] which differs in physicochemical properties compared with ALA and ABE. The stratum corneum, which is represented as a wall-like structure with corneocytes as bricks and lipid as mortar, is less permeable for a hydrophilic substance than for a lipophilic one [33]. Given the logarithm of an apparent partition coefficient ($\log P$) of ABE of 1.42315 [16], ABE is certainly more lipophilic than ALA with a $\log P$ value of -1.51692. As expected the more lipophilic ABE permeated easier through stratum corneum. Nevertheless, the barrier property of ASC is not as strong and tight as that of excised human stratum corneum as permeation data of both drugs show. ASC, which consists of a collagen gel with incorporated fibroblasts as the dermis equivalent and the upper keratinocytes layer with a few layers of cornified cells could be passed by the drugs easier than stratum corneum. Obviously cell culture techniques cannot mimic in vitro growing conditions perfectly in terms of the intercellular lipid matrix and its barrier formation.

An advantage of using ASC is the shorter running time of the permeation studies compared with stratum corneum. The permeated substances could be proven from the first hour onwards. Permeation studies across ASC were complete after one-third of the time necessary for stratum corneum. A further advantage of ASC is the lower standard error. Intra-individual variations in permeability of the stratum corneum samples resulted in a relative standard deviation of 27% for the permeation data of ALA and 25% for those of ABE, respectively. The results of the investigation with ASC showed relative standard deviations of about 9%.

The difference between the permeation data for ASC and stratum corneum is obvious. Although the difference between the permeated amounts of ALA and ABE through ASC was bigger than that through stratum corneum, ASC would be an appropriate model for screening permeation properties of ALA derivatives and of different formulations. Differences in drug permeation may be determined even more easily and with higher sensitivity. Further studies are planned to investigate the metabolism of ABE to ALA by stratum corneum, ASC, and human skin.

4. Conclusions

The permeation properties of ALA and ABE through stratum corneum and ASC as limiting step of dermal absorption could be investigated with the proposed in vitro models. The low percutaneous penetration of ALA was increased by the use of ABE. In agreement with the literature [11,12], where an increase in Pp IX concentration was observed in intact cells, ABE permeated faster through stratum corneum as well as through ASC, the latter of which was more permeable than stratum corneum for both substances. Despite less elaborated barrier functions ASC is an alternative to excised human stratum corneum to reduce the standard deviation caused by inter- and intra-individual variations of skin and to reduce the running time of the permeation studies.

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Table 2
Comparison of the permeation data for the permeation studies through ASC

Donor	Flux J (g cm^{-2} s^{-1})	Permeation coefficient P (cm/s)	Lag-time (min)	n
ALA in Excipial®-Fettcreme, 10% (w/w)	1.81×10^{-9} ($\pm 1.62 \times 10^{-10}$)	1.91×10^{-8} ($\pm 1.71 \times 10^{-9}$)	–	8
ABE in Excipial®-Fettcreme, 10% (w/w)	4.52×10^{-8} ($\pm 4.44 \times 10^{-9}$)	4.26×10^{-7} ($\pm 4.19 \times 10^{-8}$)	–	7

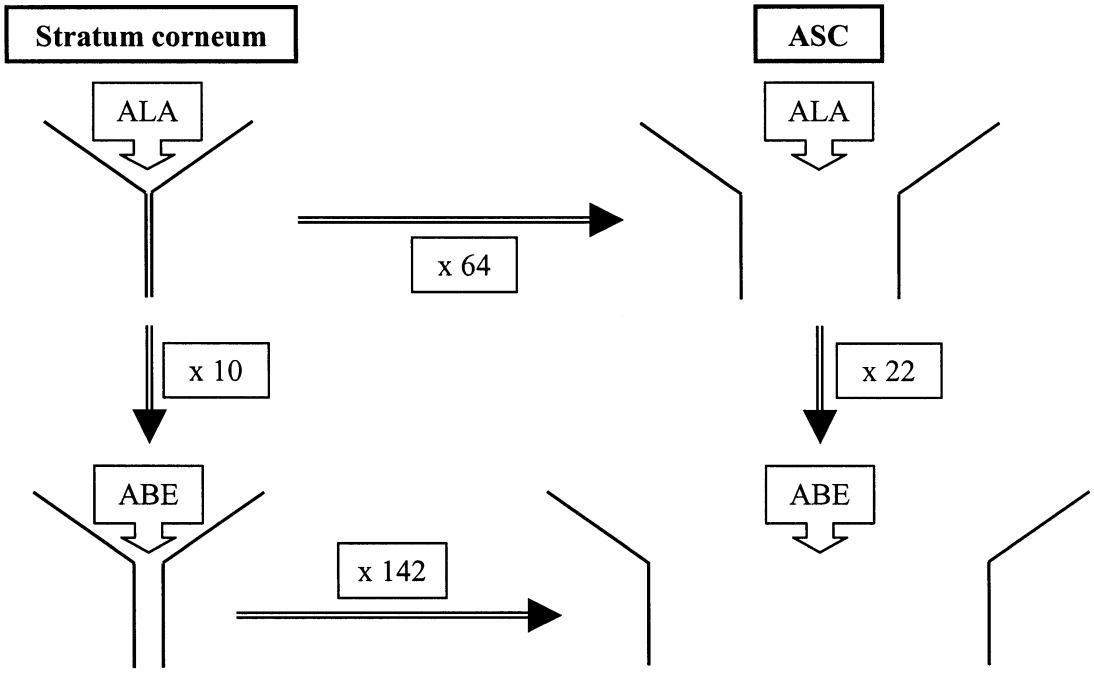

Fig. 5. Comparison of the permeation properties of ALA and ABE through stratum corneum and ASC, respectively; differences in permeability are represented by the respective diameters of the funnels.

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