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Research paper

pH profiles in human skin: influence of two in vitro test systems for drug delivery testing

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Abstract

Investigations to determine pH profiles across human stratum corneum (SC), in vivo as well as in vitro, were carried out using the tape stripping technique and a flat surface pH electrode. This method was extended to the deeper skin layers (= viable epidermis + dermis; DSL) in vitro. Statistically significant changes in the pH values were detected in the SC between in vivo and in vitro investigations and also between male and female skin in vivo. For the DSL, no gender-dependent differences in pH were observed. While the results achieved for the SC are in accordance with data already published in the literature, the values for the DSL were surprising: An alkaline pH, with a steep increase of about two pH units in the first 100 μm of the DSL and a plateau of this level was thereafter detected. Research was also done to examine the influence of different in vitro test systems on the results of pH measurements across the skin. A permeation model (Franz diffusion cell; FD-C) and a penetration model (Saarbruecken penetration model; SB-M) were compared. Experiments were carried out concerning the incubation time as well as the pH of the acceptor solution in the FD-C. Independent of the test system used, no change in the pH profiles could be observed for the SC, but a strong effect of the acceptor medium and its pH on the pH profiles across the DSL could be demonstrated using the FD-C, which showed itself partly after 30 min in statistically significant differences between incubated and formerly frozen skin. The results after the use of buffer solutions with different pH values, the pH across the DSL seemed to come into line with the one of the buffer solution, which was investigated for acidic as well as alkaline pH values. The results obtained with the flat surface pH electrode were confirmed using two different dyes: the pH-dependent fluorescent dye carboxy-SNARF-1 and the pH indicator bromthymolblue.

Keywords: pH profile; Stratum corneum; Deeper skin layer; In vitro test system; Carboxy-SNARF-1; Bromthymolblue

1. Introduction

During recent years, many investigations have been carried out concerning parameters which influence the diffusion of compounds, such as drugs, into the skin. These parameters can be divided into two groups: on the one hand, the effect of vehicles, enhancers or drug concentrations on the penetration of drugs is examined – thus, the examination specializes on factors which are dependent on the used drug formulation [1,2]. On the other hand, the characterization of the skin itself and how it may influence the penetration of drugs is investigated [3,4]. The pH in the vehicle as well as the pH across the skin are important for the diffusion of drugs, because the acid and base properties of the drug influence the solubility and partitioning in the different skin layers and therefore, the penetration of the drug. The role of varying vehicle pH values was examined by Sznitowska et al. [5] using two lipophilic drugs and concluded that within a pH range of 1.0–11.0, no effect could be observed. Concerning the pH in the skin, certain studies have focused only on the determination of the skin's surface pH [6–8]. However, in recent years, pH profiles across the stratum corneum (SC) have also been obtained [4,9,10]. Until now, no information on the pH of deeper skin layers (= viable epidermis + dermis; DSL) was available. Today, in vitro test models are widely used for the optimization of dermally applied dosage forms. According to the OECD 'No. 28 Draft Guidance Document for the Conduct of Skin Absorption Studies' [11], skin of human source is desirable for these test systems. The question arises as to whether the pH across the skin might be influenced by the conditions of different in vitro test models utilized for the examination of drug delivery to the skin. Hence, two different in vitro models were investigated – a permeation model

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and a penetration model. The results were compared with data obtained from formerly frozen skin which was not incubated in one of the test systems. The Franz diffusion cell (FD-C) [12–15] is an already well-established in vitro model to study the permeation of drugs through the skin. A liquid acceptor medium beneath the skin serves as a receptor compartment. Normally physiological buffer solutions with a pH of 7.4 are used as receptor fluid. However, depending on the drug's solubility, the addition [11] of co-solvents like ethanol or propylene glycol, or the use of different pH factors, might be necessary to maintain sink conditions during the entire experimental period. This influences the condition of the skin in a severe way. The Saarbruecken penetration model (SB-M) [16–19] can also be used to study the penetration of drugs into different skin layers. Here, the skin itself represents the acceptor for the penetrating drug. No additional liquid medium is needed. To investigate the influence of these in vitro test models on the pH in the SC and the DSL, pH profiles across the skin, in vitro as well as in vivo (only SC), were established using a flat surface pH electrode. The results obtained from the comparison of the two in vitro test systems with regard to the length of the incubation time, as well as the pH value of the used acceptor-medium, were verified using two different dyes – a pH-dependent fluorescent dye and a pH indicator.

2. Materials and methods

2.1. Materials

Multifilm kristall-klar and Fixomull (Beiersdorf, Hamburg, Germany); bromthymolblue, sodium chloride, Soerensen phosphate buffer (pH 7.4), phosphate buffer (pH 5.5 and 8.5), ammonia buffer (pH 9.0), Ringer solution, pH standard solutions (pH 4.0 and 7.0) (all components from Merck, Darmstadt, Germany); carboxy-seminaphthorhodafluor-1 (Sigma Chemical Co.; St. Louis; USA); Plastibase (Heyden GmbH, Munich, Germany); Tissue Tek (W. Plannet GmbH, Wetzlar, Germany).

2.2. Skin samples

Excised human skin from Caucasian patients, who had undergone abdominal plastic surgery, was used. The approval from the ethics committee of the 'Caritas-Traegergesellschaft Trier e.V.' as well as the consent of the patients, who were in good health and had no medical history of any dermatological disease, were available. After excision the subcutaneous fatty tissue was removed from the skin specimen using a scalpel. The skin was cut into 10×10-cm^2 pieces. Skin discs were punched out immediately for investigations with fresh skin and cleaned with water and Ringer solution. The remaining skin samples were cleaned with water, wrapped in aluminium foil, and stored in polyethylene bags at -26 °C until use. Previous studies in our laboratories had shown that under these conditions the skin is stable over a time period of 3 and 6 months with regard to drug penetration and SC thickness, respectively [20,21], which is in accordance with results from other laboratories [22,23]. For experiments using formerly frozen skin, skin discs were punched out, thawed, cleaned with Ringer solution, and investigated directly after thawing or transferred into the respective test system.

2.3. Franz diffusion cell (FD-C) experiments

In the FD-C (Franz diffusion cell type 4G-01-00-20, Perme Gear, Riegelsville, PA, USA) the skin was positioned between the donor compartment (area = 3.142 cm^2), where the drug preparation is normally contained (empty in this test series), and the acceptor compartment (volume = 15 ml), filled with Soerensen phosphate buffer, pH 7.4 (incubation times 0.5, 1, 3 and 6 h), phosphate buffer (pH 5.5 and 8.5) or ammonia buffer, pH 9.0 (incubation time 3 h). The FD-C was kept at 32 ± 1 °C by a water jacket. The acceptor fluid was mixed with a magnetic stirring bar at 500 rpm.

2.4. Saarbruecken penetration model (SB-M) experiments

Using the SB-M, the skin was transferred into the cavity of a Teflon block on filter paper soaked with Ringer solution. The latter served to minimize water loss from the skin and to prevent any change in the hydration state of the skin. Normally, the drug preparation would be filled into the cavity of a Teflon punch - in this test series it was empty. The Teflon punch was fixed in position and the gap between the two Teflon parts was sealed with Plastibase to avoid water loss from the skin. The whole apparatus was transferred into a plastic box (incubation times 3 and 6 h), which was placed in a water bath at 32 ± 1 °C.

2.5. Segmentation of the skin to obtain pH profiles across the skin

pH measurements were carried out using a flat surface pH electrode (flat surface pH electrode, InLab 426, Mettler Toledo, Switzerland; pH-meter, Knick, Berlin, Germany). The pH electrode was calibrated using two standard buffer systems before each measurement. The pH determination did not require any additional amount of liquid to be placed on the surface of the skin and was performed on the surface of the skin before the skin was placed into the respective test system. The values taken for evaluation were the ones which were constant over a time period of 15 s.

At the end of the incubation period the skin was transferred into a special apparatus, where it was mounted on cork discs using small pins, and covered with a Teflon mask with a central hole of 15 mm in diameter [18]. Thereafter, the skin was covered with 20 strips of adhesive tape (size 15×20 mm). In a standardized procedure, each tape was charged with a weight of 2 kg for 10 s and then rapidly removed [19]. The pH on the remaining skin surface was measured after every second tape strip. The tape strips were

discarded. After tape stripping, the skin was rapidly frozen in a stream of expanding carbon dioxide. A specimen with a diameter of 13 mm was taken out of the stripped area and transferred into a cryomicrotome (cryomicrotome HR Mark II, model 1978, SLEE, Mainz, Germany). The skin was cut into surface parallel sections according to the following scheme: 100, 200, 300, 400, 500, 700 and 1000 μm (the skin slices were discarded). Using a flat surface pH electrode, the pH on the remaining surface of the skin biopsy was measured. For this purpose, the skin was alternately frozen and thawed. The repeated freezing and thawing process did not influence the pH of the skin (data not shown).

2.6. In vivo experiments

Five human volunteers (three male, two female), aged 27–52 years, from whom informed consent was obtained, participated in the study. They were in good health and had no medical history of any dermatological disease. After cleaning the arms with water, a template of Fixomull with one hole was fixed on the volar left and right forearm of each volunteer. Each hole encompassed a region of 15 mm in diameter and represented one experimental area. The tape-stripping procedure and pH measurements were performed as previously described, except for exerting pressure with just the forefinger according to the recommendations recently published by the FDA and AAPS [24].

2.7. pH measurements using carboxy-seminaphtho+rhodafluor-1

A 0.5-μg/ml solution of carboxy-seminaphthorhodafluor-1 (= carboxy-SNARF-1; $pK_a \approx 7.5$) in 0.9% aqueous sodium chloride was used to verify the pH profiles of the skin after incubation in the two in vitro test systems, using a confocal laser scanning imaging system (laser scanning confocal imaging system MRC-1024, Bio-Rad Laboratories, Munich, Germany; argon ion laser, American Laser Corp., Salt Lake City, USA; Zeiss axiovert 100 microscope, Carl Zeiss, Oberkochen, Germany). Carboxy-SNARF-1 is a long-wavelength fluorescent pH indicator. The emission spectrum of carboxy-SNARF-1 undergoes a pH-dependent wavelength shift, thus allowing the ratio of the fluorescence intensities from the dye at two emission wavelengths to be used. The green fluorescence is indicative of an acidic, the red fluorescence of an alkaline pH. In the FD-C, Soerensen phosphate buffer (pH 7.4) was used as an acceptor medium. After a 1-h incubation period, the skin was frozen in a freezer and a 2 × 4-mm skin piece was cut out of the incubated area and embedded in Tissue Tek. Cross-sections (10 μm thick) were performed using a cryomicrotome. These cross-sections were stained with carboxy-SNARF-1-solution and investigated with a confocal laser scanning microscope after 30 min. The following parameters were used: 10× objective, 3% laser power, slow scan mode, gain of 1500, Kalman measurement and red/green fluorescence (filter system A1 and A2 at 488 and 514 nm).

2.8. pH measurements using bromthymolblue

A saturated solution of bromthymolblue in distilled water, filtered through a cellulose acetate filter (pore size = 0.2 μm), was used to confirm the results with the flat surface pH electrode using buffer solutions with different pHs (pH 5.5, 7.4 and 8.5) in the FD-C. Bromthymolblue changes its colour between pH 6.0 and 7.6 from yellow (acidic) to blue (alkaline). After an incubation period of 3 h, the skin was frozen in a freezer and a biopsy of 2 × 4 mm was cut out of the incubated area using a scalpel. This skin biopsy was embedded in Tissue Tek and transferred in a cryomicrotome. Cross-sections 40 μm thick were obtained and coloured with the bromthymolblue solution. Light microscopic investigations (light microscope Olympus BH-2, Olympus, Hamburg, Germany) were performed directly after staining.

3. Results

The in vitro pH experiments were investigated with a minimum of two separate donor skin flaps, and up to four, if enough different skin donors were available (exception: in vitro investigations with male excised human skin - only one skin flap was at hand). Two or three replicates per experiment were carried out.

3.1. pH profiles across human stratum corneum (SC)

Fig. 1a and b (separate for better clarity) demonstrate pH profiles across human SC in vivo and in vitro as a function of gender and storage (excised skin). A more acidic pH can be detected on male skin (Fig. 1b) in comparison with female skin (Fig. 1a) in vivo as well as in vitro. With increasing SC depth, the pH rises slightly in most of the shown profiles, but only by around half a pH unit. The differences between the in vitro results – fresh skin versus formerly frozen skin – are only small, indicating that the freezing process hardly influences the pH of the skin. On the other hand, when comparing each SC depth on its own, using the same number of tape strips, the pH values showed statistically significant differences (*t*-test, Sigma Plot 4.01; $^{\circ}P < 0.05$ and $^{*}P < 0.01$) between the in vitro and in vivo results independent of sex. Between male and female skin under in vivo conditions significant differences are also found with $P < 0.01$ up to the 12th strip and $P < 0.05$ up to the 16th strip.

3.2. pH profiles across human deeper skin layers (DSL)

Fig. 2 shows pH profiles across the DSL as a function of gender. Only in vitro results were available. Independent of gender and storage of the skin, the same tendency can be observed in all cases: a steep increase of the pH around 2 pH

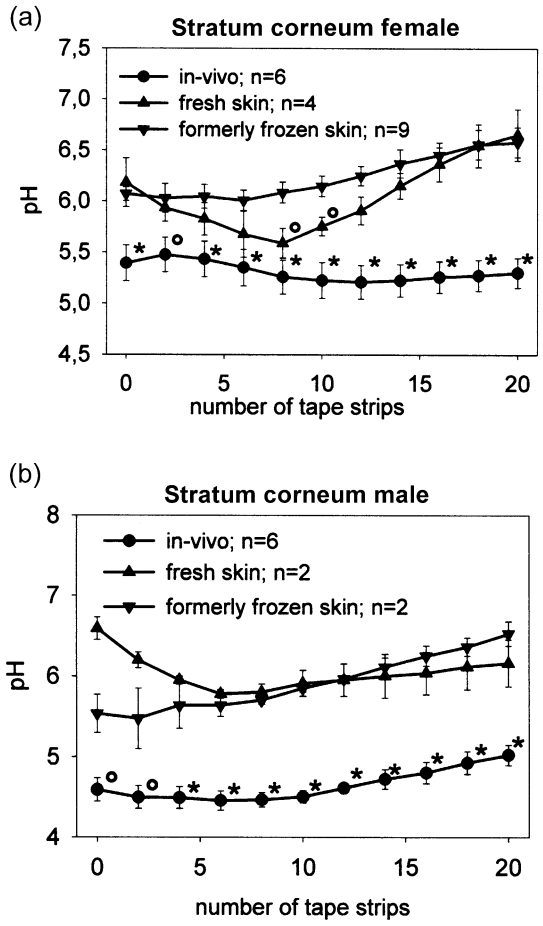

Fig. 1. pH profiles across human stratum corneum in vivo and in vitro (pH value ± SE versus number of tape strips; 1–4 skin flaps from different donors with 2–3 replicates each; statistically significant differences are marked with $^{\circ}P < 0.05$ and $^{*}P < 0.01$).

units from an acidic to an alkaline pH in the first 100 μm after the removal of the SC. For different skin depths, no statistically significant differences can be detected for fresh or formerly frozen skin, neither for female nor male skin (*t*-test; Sigma Plot 4.01).

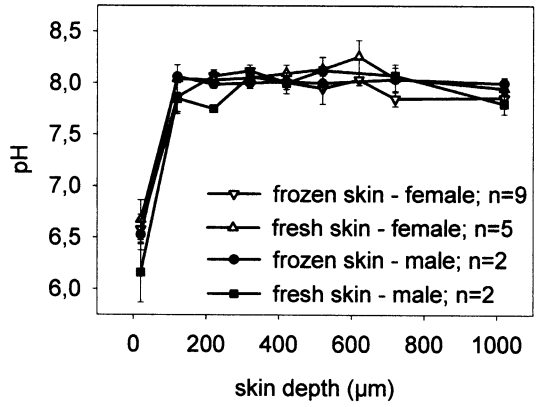

Fig. 2. pH profiles across human deeper skin layers in vitro (pH value ± SE versus skin depth (μm); 1–4 skin flaps with 2–3 replicates each).

3.3. Influence of the conditions of the in vitro test system: incubation time

The main difference between the investigated in vitro test systems is the hydration rate of the skin. While the water content of the skin is more or less unaffected in the SB-M, water uptake from the basolateral side can be observed in the FD-C [19]. pH measurements after different incubation times were carried out to investigate whether the in vitro test conditions influence the pH of the skin. Fig. 3 indicates the results in the SC after 0.5, 1, 3 and 6 h incubation in the FD-C (acceptor medium = Soerensen phosphate buffer solution, pH 7.4), and after 3 and 6 h incubation in the SB-M in comparison with formerly frozen skin. Although slight differences can be observed for the different incubation periods in both test systems, these differences are not statistically significant (*t*-test, Sigma Plot 4.01) for the SC regarding single SC depths, i.e. number of tape strips. Fig. 4 shows the results for the DSL for the same experimental design. Using the FD-C, the pH in the skin decreased with increasing incubation times. This is statistically significant ($^{*}P < 0.01$), especially for some of the investigated skin

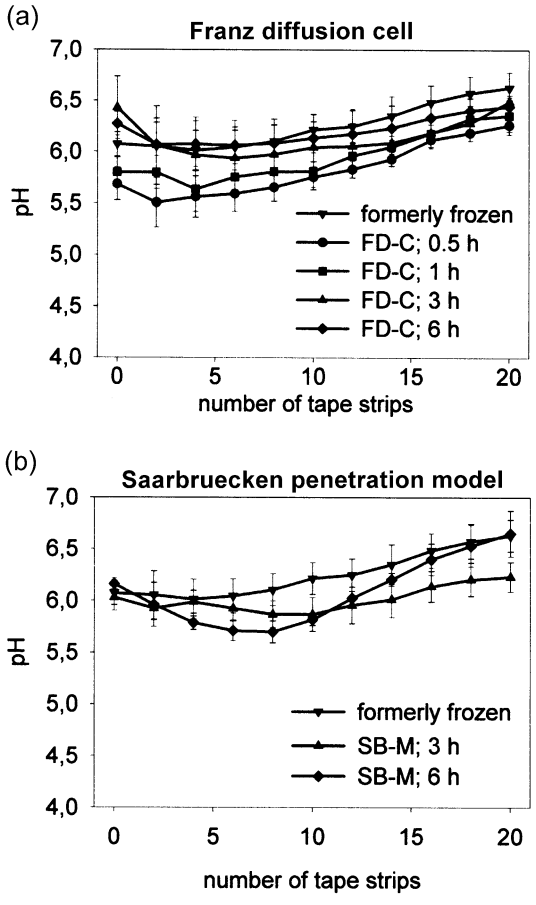

Fig. 3. pH profiles across human stratum corneum: comparison between the Franz diffusion cell (acceptor medium pH 7.4) and the Saarbruecken penetration model (no liquid acceptor medium) (pH value ± SE versus number of tape strips; two skin flaps with 4–5 replicates each).

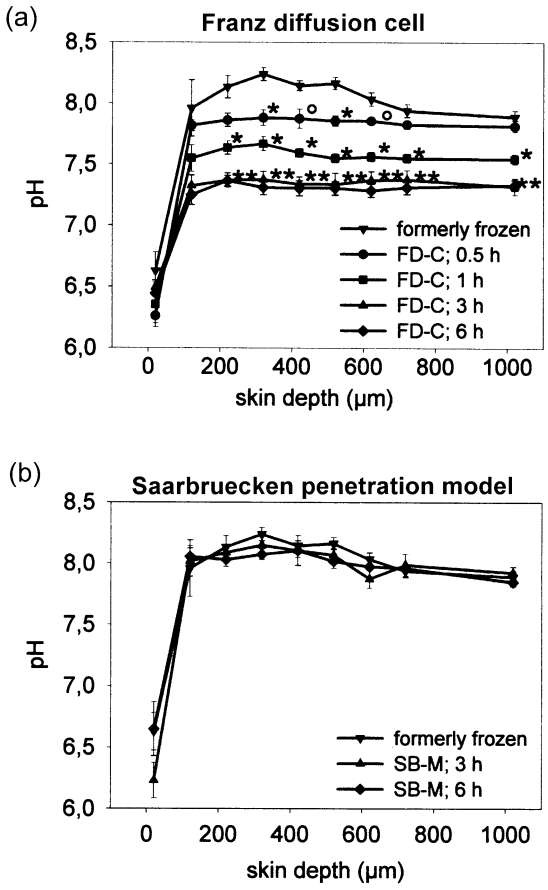

Fig. 4. pH profiles across human deeper skin layers: comparison between the Franz diffusion cell (acceptor medium pH 7.4) and the Saarbruecken penetration model (no liquid acceptor medium) (pH value ± SE versus skin depth (µm); two skin flaps with 4–5 replicates each; statistically significant differences are marked with $^{\circ}P < 0.05$ and $^{*}P < 0.01$).

depths after 0.5 h incubation. After 3 and 6 h incubation, the skin's pH is more or less the same as the one of the acceptor medium. In the SB-M, no change of the pH of the skin is detectable, although only the longer incubation periods were investigated.

3.4. Influence of the conditions of the in vitro test system: confirmation with the use of a fluorescent dye

To confirm the results obtained with the flat surface pH electrode after the skin's incubation in the FD-C (acceptor medium = Soerensen phosphate buffer, pH 7.4) and the SB-M, the pH-dependent fluorescent indicator carboxy-SNARF-1 was used to stain cross-sections of the skin after 1 h incubation in the respective test system. Fig. 5 demonstrates the results. In both cases, a green fluorescence is detected in the SC which confirms the acidic pH measured with the flat surface pH electrode. A red fluorescence in the epidermis is also observed using both test systems, indicating a more alkaline pH, which is in accordance with the steep pH increase of 2 pH units measured in a skin depth of 100 µm with the flat surface pH electrode after tape stripping. Differences are measured in the dermis. The green fluorescence which can be observed after the use of the FD-C, in comparison with the red fluorescence, which is detectable in the skin mounted in the SB-M, indicates a lower pH value of the DSL after experiments in the FD-C. These results are in agreement with the data obtained using the flat surface pH electrode (compare with Fig. 4: FD-C (1 h), pH 7.6; SB-M (3 h), pH 8.0) after cryosectioning.

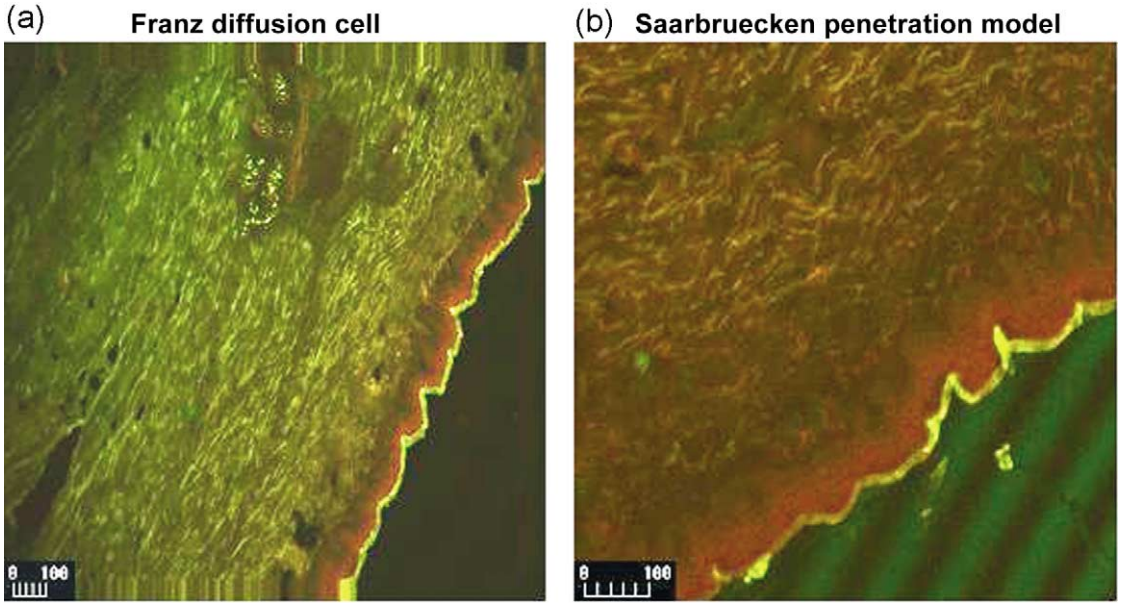

Fig. 5. Comparison of the Franz diffusion cell with the Saarbruecken penetration model: cross-sections of the skin coloured with carboxy-SNARF-1 (1 h incubation time/confocal laser scanning microscopy).

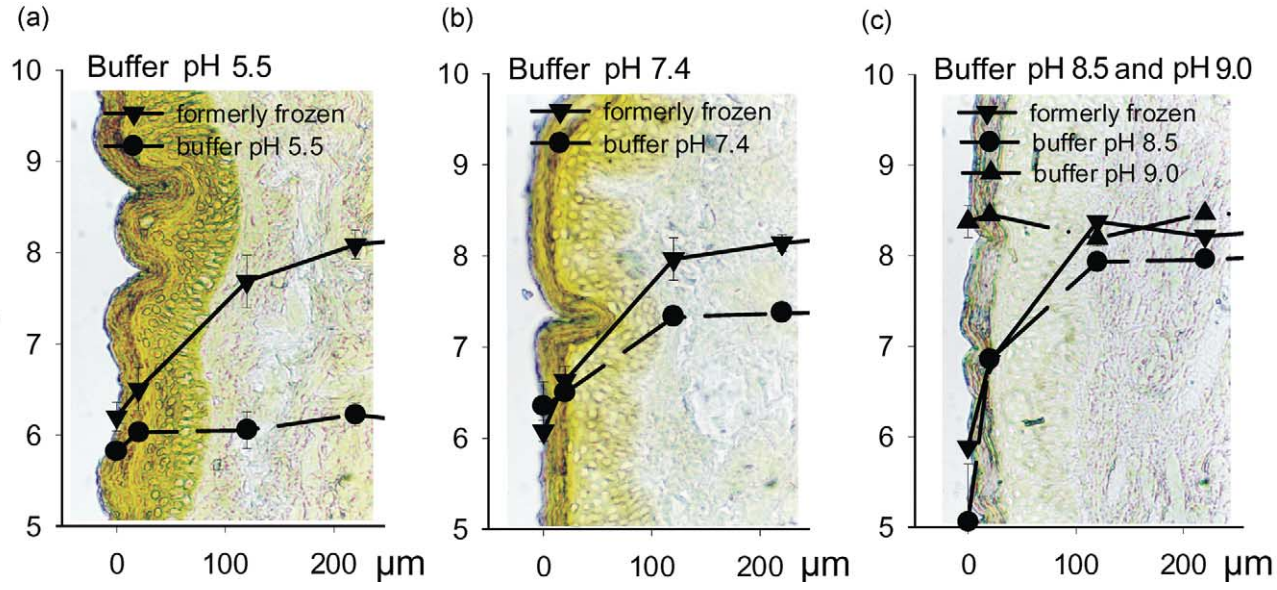

Fig. 6. Influence of the pH value of the acceptor medium in the Franz diffusion cell: cross-sections of the skin stained with bromthymolblue/verification with pH profiles – 3 h incubation time (pH value ± SE versus skin depth (µm); two skin flaps with two replicates each).

3.5. Influence of the conditions of the in vitro test system: pH of the acceptor medium

Fig. 6 illustrates pH profiles across excised human skin after the use of various buffer solutions with different pHs as acceptor medium in the FD-C (pH 5.5, 7.4, 8.5 and 9.0) in comparison with formerly frozen skin. After an incubation period of 3 h, the pH in the DSL comes more or less into line with one of the buffer solutions, but obviously the buffer capacity of the skin with regard to an alkaline pH seems to be higher than the one for an acidic pH. In accordance with the other results, the pH in the SC is acidic in all three experimental designs, with one exception: using the ammonia buffer pH 9.0, the pH in the SC was also alkaline. Experiments with the pH indicator bromthymolblue, which can also be seen in Fig. 6 (buffer solutions with pH 5.5, 7.4 and 8.5), confirm the results of the pH profiles. Using buffer pH 5.5 and 7.4, the SC and the epidermis are stained yellow, indicating an acidic pH. The DSL are stained yellow, too, using buffer pH 5.5, and coloured light blue using buffer pH 7.4. While the yellow colour also indicates an acidic pH, the light blue colour represents a neutral to alkaline pH. Using buffer pH 8.5, the DSL are stained blue, corresponding to an alkaline pH.

4. Discussion

Two major subjects have been investigated in this study: the pH in human skin in vivo (only SC) as well as in vitro, and the effect of the acceptor phase of different in vitro test systems on the pH across skin specimens.

4.1. pH profiles across human SC and DSL

In vivo pH measurements across the SC shown in Fig. 1 using the tape-stripping technique have been reported previously [4,9,10]. Dependent on the laboratory, the number of tape strips and the type of adhesive tape used differed and the skin source (human or animal) varied, but an acidic pH was always found for the SC. For example, Oehman et al. [4] described two different methods to determine pH values across the SC in vivo: one using a cellophane tape (stripping 100–120 times) and a second using cyanoacrylate resin (stripping 10 times). They found an increasing surface pH with rising numbers of strips for both men and women in vivo, with a sigmoidal curve for the pH values obtained from men. Our data also illustrate an increasing pH value across the SC, but the sigmoidal curve could not be confirmed in vivo, only in vitro, using formerly frozen skin independent of gender. In our study, as well as in the one of Oehman et al. [4], a gender-dependent pH profile was detected in vivo. Male skin was more acidic than female skin. It may be speculated that the different pH values obtained may be caused by women's frequent use of cosmetics [25].

Using excised human male or female skin, no matter whether the investigations were carried out directly after excision (fresh skin) or after storage in the freezer (formerly frozen skin), increased pH values were obtained as compared to the in vivo pH data. The reason for this fact is not fully known. Two possibilities are conceivable. Firstly, different anatomical sites for the in vitro and in vivo investigations (abdomen for in vitro; forearm for in vivo) were used. It is well known that not only the skin's surface pH [6–8], but also the lipid composition in the SC

[26–29], differ as a function of the skin region, which could lead to changes in the pH profile across the SC. Secondly, the season in which the pH determinations were carried out could also play a role. Investigations concerning this parameter [30] showed that during summer the pH of the skin surface is 0.5 pH units below the pH values during the rest of the year. Due to the reason that the in vivo experiments were carried out in July, but the excisions of the used skin flaps took place in October, January and April, differences between in vivo and in vitro data may be increased.

For the DSL, only in vitro data were available (illustrated in Fig. 2). In correspondence with the in vitro SC data, no significant differences could be detected between male and female skin and fresh and formerly frozen skin, respectively. In contrast to the SC, an alkaline pH of around 8.0 was determined at a skin depth of 100 μm and higher. This was astonishing, as it was expected to measure a pH value of around 7.4, which was found in the epidermis using cyanoacrylate resin [4] and which is comparable to the pH of blood. An interpretation of this result is difficult; necrotic reactions may have occurred. An attempt was made to find an explanation with the help of the pH optima of several enzymes, localized in the skin, which were mentioned in the literature [31–35]. Most described enzymes (localization in the SC and epidermis) are known for their acidic pH – a fact that confirms the results detected in the SC – while enzymes with a pH optimum of 8.0 were also found in the SC [36,37]. Our result may be an indication that pH values around 8.0 actually exist in the skin. All listed data resulted from experiments under viable conditions of the concerned tissue fraction. Unfortunately, information about the effect of excision on the characteristics of the skin, especially the dermis, is still lacking.

4.2. Influence of the in vitro test system

Several different in vitro test systems are used to study the diffusion of drugs into the skin. The FD-C, which was one of the test systems investigated in this study, is a permeation model established to obtain information about percutaneous absorption of drugs. To determine the effect of the acceptor medium beneath the skin, investigations concerning the influence of the osmotic strength of, or the addition of antimicrobial agents in the acceptor medium, have already been carried out [38,39]. Until now, a consideration of the importance of the pH of the acceptor medium has been lacking, and its influence on the incubated skin specimen and drug diffusion was unknown. This information may be of interest if the permeation experiments are to be carried out with a drug insoluble in the normally used buffers of pH 7.4. In such a case, varying buffer systems with differing pH values and/or organic solvents are necessary [11].

The pH profiles illustrated in Fig. 4 demonstrate the influence of the acceptor medium on the pH of the DSL in a convincing manner. While no differences in the pH values could be observed between incubation in the SB-M and formerly frozen skin, the pH value using the FD-C decreased with incubation time until it reached the pH of the acceptor fluid after 3 h. This knowledge is very important with respect to the usually performed pre-incubation of the skin with the acceptor medium, which normally lasts several hours. Thereafter an actual permeation experiment is carried out, possibly with a skin flap of totally different properties, because a change of the skin's pH is linked with a change of the solubility of the drug within the various skin layers.

These data were confirmed using a pH-dependent fluorescent dye, carboxy-SNARF-1 (Fig. 5). Up to now, several attempts have been made to find a suitable method to measure the intracellular pH in isolated cells [40–42] by the employment of fluorescent dyes. Although this technique could be used successfully in cell cultures, some problems arise when transferring this method to the skin [10]. In our study, the determination of the pH was made only by colouring.

FD-C experiments carried out using buffer solutions with an acidic, a more or less neutral and two different alkaline pHs showed that it is possible to shift the pH of the skin in every direction, acidic as well as alkaline. Fig. 6 demonstrates the results which were confirmed with the pH indicator bromthymolblue. The effect is not significant for the SC. However, for the DSL, significant differences in the pH are visible in comparison with formerly frozen skin when using a buffer solution with acid or neutral pH. After carrying out the experiments with an ammonia buffer, the SC became alkaline as well, which is significantly different from non-incubated SC, caused perhaps by the volatility of the ammonia, which could be smelled during the stripping procedure.

5. Conclusions

The results of this study demonstrate that the conditions of the in vitro test systems influence the pH in the skin, which, for its part, will influence the diffusion of drugs and their distribution in the different skin layers. On the one hand, depending on the acidic or alkaline properties of the investigated drug, its solubility will differ completely from the one under in vivo conditions. On the other hand, components of the acceptor medium with a high volatility, can cause a shift of the pH not only in the DSL but also in the SC and therefore, a change in the ionization degree of the fatty acids within the SC can occur, which may lead to a reorganization of the SC lipids and will also affect the permeation of drugs [43,44]. As a result, it may be necessary to reconsider the use of such aqueous acceptor media and to be more critical about the results obtained from these in vitro test systems.

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