Influence of Drug Treatment on the Microacidity in Rat and Human Skin—An *In Vitro* Electron Spin Resonance Imaging Study

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Purpose. The possibilities of the noninvasive examination of microacidity[5] in different depths of the skin *in vitro* was explored, and the impact of drug treatment on the pH inside the skin was studied.

Methods. Spectral-spatial electron spin resonance imaging (ss-ESRI) and pH-sensitive nitroxides were used to obtain a pH map of rat and human skin *in vitro*.

Results. The dermal application of therapeutically used acids, such as salicylic acid and azelaic acid, caused a plain change of microacidity (pH) inside the skin. Species-linked differences between rat and human skin samples with respect to penetration and microacidity were found.

Conclusions. ESRI has been shown to be a new and completely noninvasive method to monitor microacidity in different skin layers and on the skin surface. This nondestructive method allows serial measurements on skin samples to be performed without any preparatory steps.

KEY WORDS: skin pH; ESR imaging; skin penetration; pH gradients.

INTRODUCTION

Skin pH is an important factor for the evaluation of pharmacological and toxicological effects of drugs and xenobiotics. There are a large number of common and extensively used drugs in dermatology that are based on acids or bases. Moreover, the chronic alkalization by soaps, detergents, or surfactants is damaging to the skin and is under discussion. It has been shown that skin alkalization is correlated with high transepidermal water loss and a low stratum corneum hydration (1).

Most research groups have used planar glass pH electrodes, as well as pH transistor technology electrodes, to investigate the microacidity on the skin surface (2). Techniques based on nuclear magnetic resonance, such as ^{31}P-NMR- (3) and ^{19}F-NMR-techniques (4) permit pH measurements inside the skin, but the low sensitivity and accuracy of these NMR-techniques are disadvantageous (5).

The possibilities of accurate determination of pH values by ESR are based on the findings of Khramtsov that the hyperfine splitting constant a_N of imidazoline and imidazolidine nitroxides depends on the pH of the surrounding solution (6,7). About 1.5–2 pH units above and below the pK_a value of the nitroxides it is possible to use the splitting constant a_N as a very sensitive pH indicator. Monitoring changes of microacidity caused by drug decomposition in aqueous droplets of nontransparent w/o ointments had been one of the first applications of the pH measurement by ESR (8). ESR has also been used to measure the influence of incorporated drugs on the microacidity in microparticles (9) and tablets (10). In recent investigations, Gallez and Mäder provided a new noninvasive method to measure pH *in vivo* inside degrading poly-lactide-co-glycolide implants (11) and the gut (12) using pH-sensitive nitroxides and low frequency ESR spectroscopy at 1.1 GHz.

ESR imaging (ESRI) provides information about the spatial distribution of paramagnetic compounds (e.g., nitroxides) in a heterogenous sample. This method is based on recording a set of ESR spectra under the influence of well-defined field gradients. After deconvolution and image reconstruction, statements about the spatial distribution of paramagnetic centres (e.g., nitroxide spin probes) in the examined object can be made. The combination of spectral information and spatial distribution of paramagnetic centres in the method of spectral-spatial ESR Imaging (ss-ESRI) developed by Maltempo allows the detection of property changes in different parts of an object (13).

It has also been demonstrated recently that ESRI permits the slice-specific determination of microacidity. It was found for the first time that pH gradients of 2 pH units within a distance of few microns emerged with time in degrading polyanhydrides (14). These results encouraged us to think about the possibilities to monitor the microacidity in different depths of skin biopsies by applying a pH-sensitive nitroxide and the method of ss-ESRI. Moreover, spectral-spatial ESRI was applied to investigate the influence of drug treatment on the microacidity in human and rat skin *in vitro* in order to monitor the impact of drugs on the microacidity of distinct skin layers.

MATERIALS AND METHODS

Materials

The nitroxide spin probes 4-amino-2,2,5,5-tetramethylimidazoline-1-yloxy (AT) and 2,2,3,4,5,5-hexamethylimidazolidine-1-yloxy (HM) were purchased from the Institute of Organic Chemistry (group of Prof. Grigoriev), Russian Academy of Science, Novosibirsk, Russia. DPPH was obtained from BDH Chemicals Ltd., England and coal was a self-made sample of charred saccharose (600°C, absence of oxygen). Rheuma-Salbe STADA® (containing 5% 2-hydroxyethylsalicylate) was purchased from STADApharm GmbH/Germany and Skinoren® Creme (containing 20 % azelaic acid) was obtained from Asche AG/Germany. All other chemical were of highest purity available.

Methods

Experimental Settings for the ESRI Studies

The ESR spectra were recorded using an X-band spectrometer ERS 220 (ZWG, Berlin-Adlershof, Germany) with

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[5] In this article, we use the terms microacidity and pH value for our systems despite being conscious about the term pH value which is defined for aqueous systems only.

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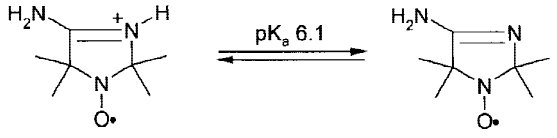
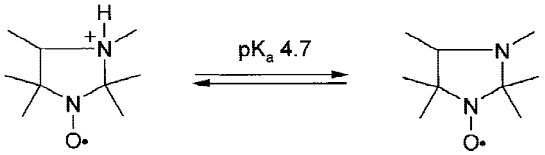


4-Amino-2,2,5,5-tetramethylimidazoline-1-oxyl (AT)



2,2,3,4,5,5-Hexamethylimidazolidine-1-oxyl (HM)

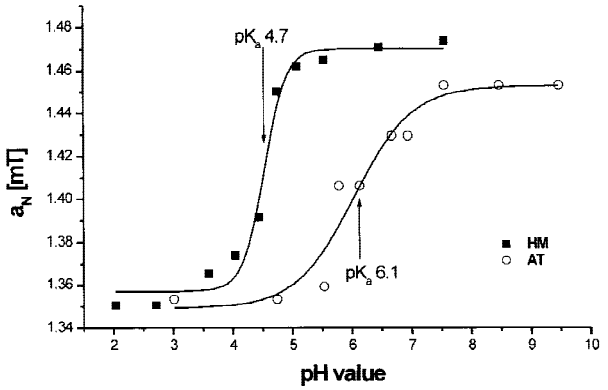


Fig. 1. Coupling constants a_N of the spin probes AT and HM in dependency on the pH value of the aqueous solution (lines: sigmoidal Boltzmann fit).

a home-made imaging extension. The biopsies have been placed into a modified tissue cell (minimal air-volume, integrated α-diphenylpicrylhydrazyl [DPPH] standard serving as intensity and spatial standard) and measured in a common H_{102}-cavity. The following settings were used: microwave frequency 9.35 GHz; microwave power 2 mW; modulation amplitude 0.1 mT; scan width 12 mT; maximum gradient 3.84 T/m; scan time per projection 10 s; 95 projections; 512 points per projection; image reconstruction: filtered backprojection giving an image matrix of 256 × 256 points. The temperature inside the resonator was set in a range of 20–37°C. The distribution of nitroxide spin probes was quantified using the signal amplitude of the middle field peak.

pH Calibration

The pH-sensitive nitroxides were dissolved in Britton-Robinson-buffer (preparation according to H. M. Rauen, reference 15) giving a final concentration of 1 mM. The pH values were adjusted by adding 0.1 N NaOH and 0.1 N HCl, respectively, and checked using a glass electrode (WTW pH 522 Weilheim/Germany with a planar glass electrode Mettler Toledo InLab® 424) and commercial reference buffers (pH buffer solutions, Mettler Toledo Steinbach/Germany). One hundred microliters of the Britton–Robinson buffer solution

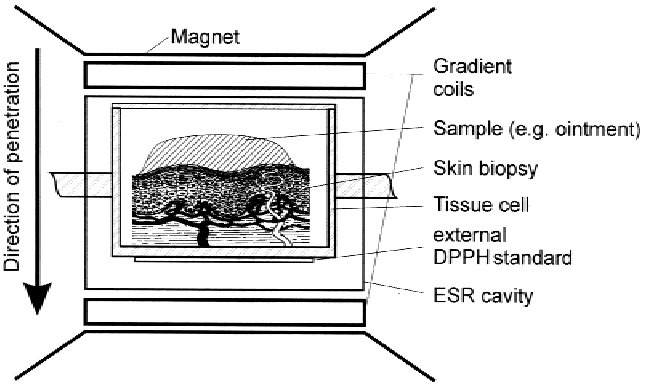


Fig. 2. Sample arrangement for spectral-spatial ESR tomography studies of excised animal and human skin (surface area of skin biopsies: 1.1 × 2.5 mm^2 taken with a pair of standard biopsy scissors; sample volume: 1–1.5 μl; temperature range 20–37°C; dimensions in the sketch are not scaled).

were filled in a quartz flat cell and transferred to the spectrometer immediately and ESRI measurements were carried out. The hyperfine coupling constants a_N of the integrated spectra have been taken for establishing the calibration curve. For pH measurement in skin two solutions have been prepared. First, a 1-ml solution of the pH-sensitive nitroxide HM (10 g/L) in deionized and distilled water was made. Deionized and distilled water was used to avoid changes in pH values and composition. Second, a 1-ml solution of the nitroxide AT (10 g/L) in microemulsion (30% water, 24.15% Tween 80; 7.245% dodecanol, 7.245% Cera perliquida, and 31.36% propylenglycol) was prepared.

Skin Preparation

The human skin (abdomen and thigh female skin) was obtained from cosmetic surgery. The fat tissue was carefully removed, and the skin was cleansed. For rat skin biopsies, male Wistar rats were purchased from Tierzucht Schönwalde GmbH/Germany. The rats were decapitated, shaved, and a skin sample from the back was used for the experiments. Finally, the skin samples were cut into pieces (about 1 cm^2 area) and kept on agar plates (medium: 199 Earle/Bioderm KG with agar 0.01 g/ml) at 4°C until measurement (48 hours maximum).

pH Measurements

To study the microacidity, a 5-μl solution containing the pH-sensitive nitroxide were applied to the skin surface. The skin samples were again stored on agar plates at 35°C and

Table I. Comparison of the Hyperfine Splitting Constants a_N (in mT) of HM in Dependence on the Solvent Properties

	a_N [mT]
HM in aqueous solution at pH 2.03	1.466
HM in aqueous solution at pH 7.55	1.594
HM in neutral oil	1.488

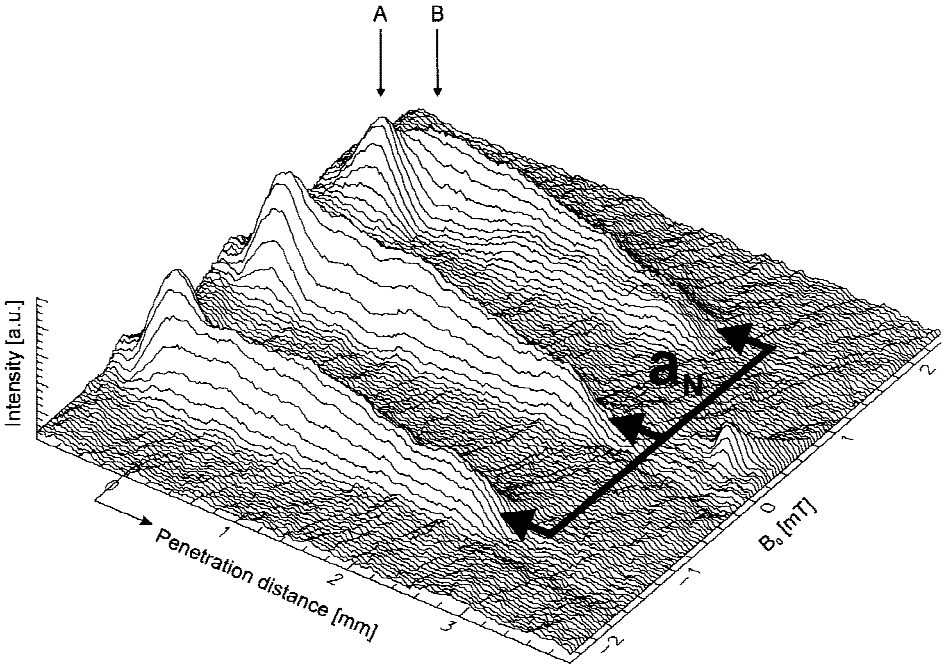


Fig. 3. ESR tomogram of the human skin. A skin biopsy (pretreated with the pH sensitive spin probe HM) 40 minutes after the application of salicylic acid (5% solution in microemulsion; preparation A); the hyperfine coupling constant a_N indicates the high microacidity in the surrounding of the spin probe on the skin surface respectively inside the upper skin layers (A) in contrast to the lower dermis (B; spectral-spatial plot; the tiny signal on the right-hand side is that of the DPPH sample which serves as spatial and intensity standard).

75% rel. humidity for 30 minutes further. After that the residue of the solution was carefully removed from the skin, a biopsy was taken and the sample was applied onto the skin surface. The following preparations were used to affect the microacidity inside the skin probe: preparation A (salicylic acid in 5% microemulsion); preparation B (2-hydroxyethylsalicylic acid 5% in creme, commercially available as Rheuma-Salbe STADA®); preparation C (azelaic acid 20% in creme, commercially available as Skinoren® Creme).

The biopsies were kept under the conditions described above and after fixed periods of time (30 minutes), a set of ESR spectra was recorded. The thickness of the skin

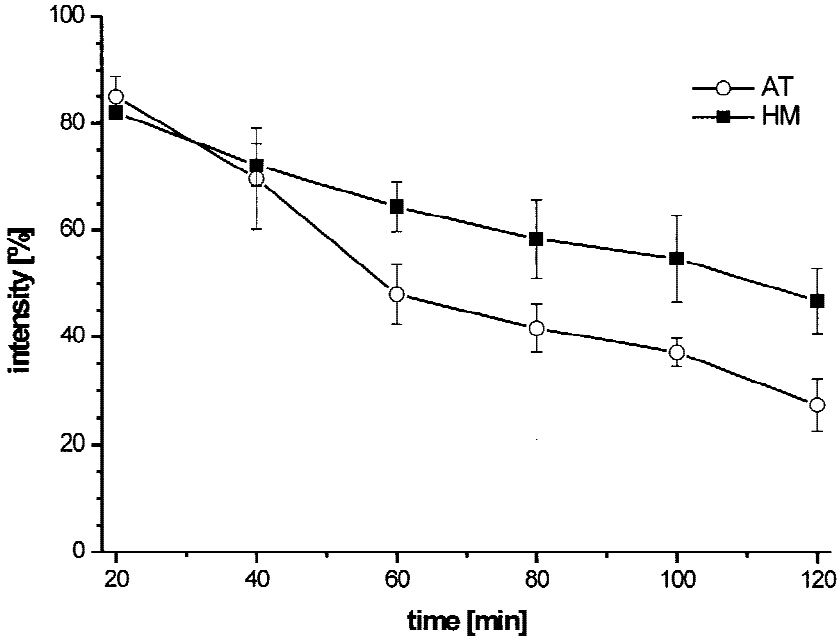


Fig. 4. Decrease of signal intensity of the spin probes AT and HM in dependency on the time after application (determined by comparing the signal intensity with that of the external DPPH standard).

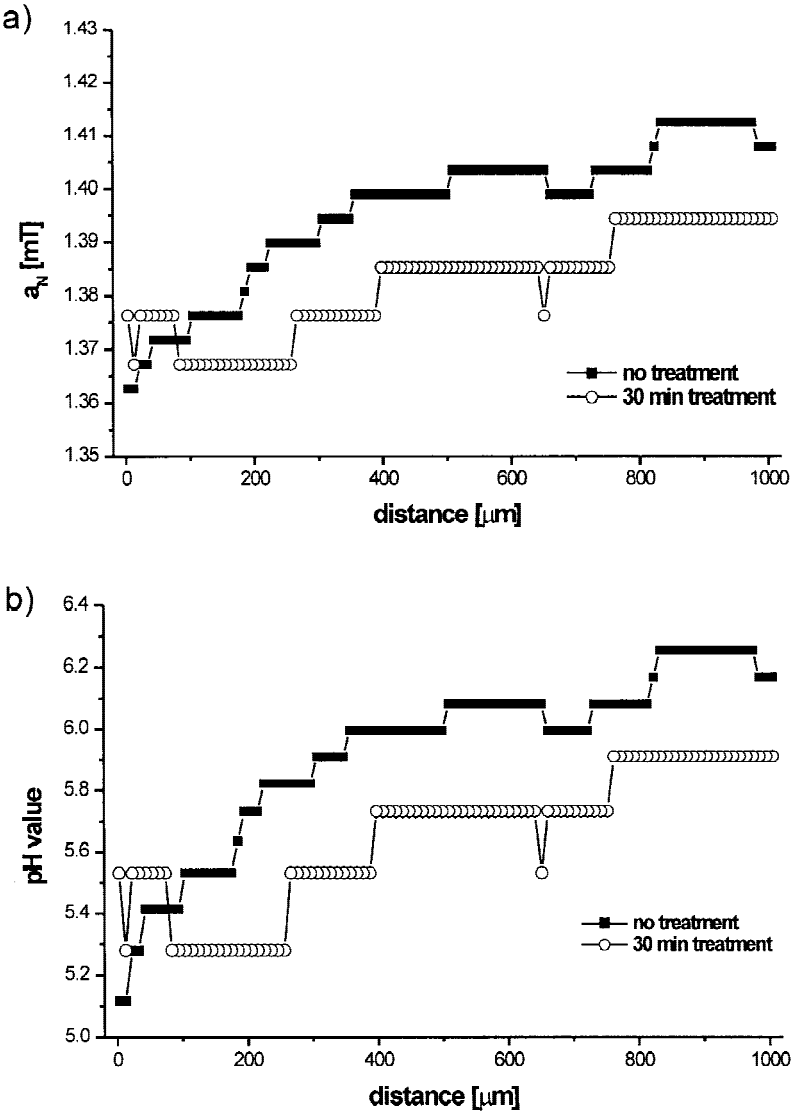


Fig. 5. Change in the hyperfine splitting constant a_N of the pH-sensitive spin probe AT (a) and microacidity (b) inside a human skin biopsy as the result of treatment with salicylic acid (5% in microemulsion); the microacidity has been calculated using a calibration curve (see Fig. 1 for details).

was determined using the external DPPH standard and a sample of charred saccharose placed on the skin surface.

RESULTS AND DISCUSSION

The ESR measurements of the pH-sensitive nitroxides AT and HM show the dependence of the hyperfine coupling constants a_N on the pH value in aqueous solution and microemulsion (which contains 30% water). The calibration curve was established by a sigmoidal Boltzman fit to the hyperfine coupling constants. The obtained data were in good agreement with earlier investigations using ESR spectroscopy (8) and the specifications for AT and HM in the literature (6). The results are presented in Figure 1.

Figure 2 illustrates the arrangement of biopsy and sample within the cavity. The biopsy was placed on a quartz sheet opposite to the above-mentioned DPPH standard. The quartz sheet was fixed to the modified tissue cell and than placed in the H_{102} cavity with the sheet perpendicular to the direction of the field gradient.

In the initial study, the penetration of nitroxide spin probes into skin biopsies of human and rat skin was investigated. As expected, aqueous solutions of nitroxides showed a rapid penetration into the skin biopsies from rat skin. Human skin samples showed a different behavior toward the nitroxides HM and AT. The more lipophilic nitroxide HM revealed a high penetration rate, whereas the concentration of hydrophilic AT was too low for recording ESR tomograms of the skin biopsies. The addition of DMSO (1, 2, and 5%) did not enhance the penetration rate significantly. Because of this, the highly hydrophilic nitroxide AT was dissolved in microemulsion for all further ESR experiments. In Figure 3, the distribution pattern of the pH-sensitive nitroxide spin probe HM in a human skin biopsy (30 minutes pretreatment) is shown 40 minutes after administration of salicylic acid (preparation A) on the skin surface. The signal intensity corre-

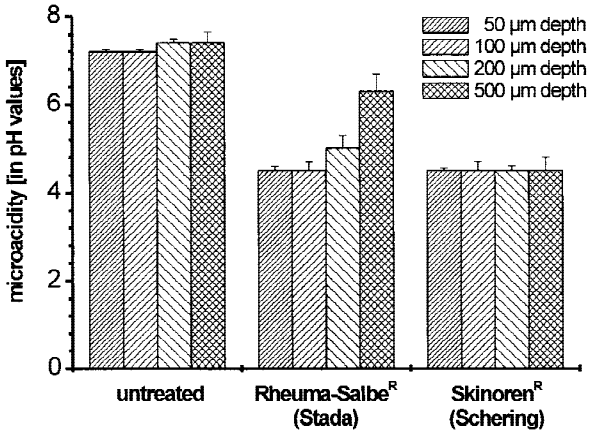


Fig. 6. Impact of 2-hydroxyethylsalicylate (Rheuma-Salbe STADA®) and azelaic acid (Skinoren® Creme).

sponds to the concentration of the nitroxide inside the skin. At this time, a complete but not uniform distribution of HM into the whole skin biopsy was observed. At the end of the period of measurements, the concentration of HM in the deeper layers of the skin was higher than the remaining concentration in the upper layers. These results were in good agreement with the partition coefficient (1.6 in heptane/buffer at pH 7.4) (16). No differences between rat skin and human skin could be detected for the penetration of HM. After application of salicylic acid on human skin surface, the microacidity increased on the skin surface and into upper layers of the epidermis, as is observed from the decreasing hyperfine coupling constant a_N of HM in Figure 3. The extent of the pH-gradient inside the sample depends on the flux of salicylic acid into the skin. In the case of HM, difficulties of the pH determination may arise from the lipophilicity of the spin probe. In the presence of lipid rich tissue, the probe may have three different hyperfine coupling constants due to the presence of the following three species: a) aqueous environment, protonated; b) aqueous environment, non-protonated; and c) lipophilic environment (Table I). Artifacts may arise because of the small difference of the hyperfine coupling between species a and c. This problem does not exist for the hydrophilic probe AT.

The penetration flux of the hydrophilic nitroxide AT dissolved in pure water is very low in human skin (partition coefficient of 0.08 in heptane/buffer at pH 7.4). Furthermore, inside the skin nitroxides are partially reduced by several enzymatic and nonenzymatic mechanisms to diamagnetic (ESR silent) products (17). Thus after application of an aqueous solution of AT, the remaining concentration of the spin probe inside the skin was too low for ESRI investigations. To determine the microacidity inside the skin, it was necessary to increase the concentration of the water solubilized AT molecules. For that reason, AT was dissolved in the microemulsion or in a DMSO solution (10% DMSO in water), respectively, to enhance the penetration rate into the skin biopsy. Nitroxide penetration was faster on skin treatment with a microemulsion than with DMSO solution.

The concentration of the spin probes in the skin biopsies had been sufficiently large to record tomograms over the complete period of measurements (Fig. 4). The reduction of the respective spin probe might depend on the individual, the place of sampling, sample preparation, and the age of the biopsy.

Furthermore, the penetration of the nitroxide spin probe AT between human and rat skin showed remarkable differences. The penetration velocity of AT was more than 5–10 times higher in rat skin than in human skin. This phenomenon can be explained by a polar penetration route due to the larger number of hair follicles of the rat skin. As a conclusion from that it can be stated that the penetration of hydrophilic nitroxides is much faster in the upper rat skin epidermis than in the human stratum corneum.

In Figure 5a, the hyperfine coupling constant a_N of the nitroxide spin probe AT is shown to be dependent on the depth of the human skin. The treatment of the skin biopsy with acidic drugs such as salicylic acid caused a gradual change to lower coupling constants in dependency on time, penetration depth, and pharmaceutical formulation, respectively. The microacidity (Fig. 5b) inside the skin biopsy was calculated using the calibration curve of Figure 1. A distinct decrease of microacidity was observed from the upper parts of the skin (stratum corneum) to the inner layers of the dermis. This phenomenon can be explained by the extent of penetration of salicylic acid into the biopsy and the relative high buffer capacity of the skin.

Figure 6 shows the microacidity in depths of 50, 100, 200, and 500 µm after treatment of the skin surface with 2-hydroxyethyl-salicyliate acid (Rheuma-Salbe STADA® containing 5 % 2-hydroxyethyl-salicylate) and azelaic acid (Skinoren® Creme containing 20 % azelaic acid) for 30 minutes. Both commercial preparations are widely used. Application of these drugs caused a gradual change to lower pH values in the epidermis and dermis of the skin. The existence of a pH gradient inside the skin biopsy was dependent on chemical structure, biophysical behaviour, and the stability of the drugs. As expected, the highly lipophilic and long-chained molecule of azelaic acid in preparation C, containing two free carboxyl groups (nonanedioic acid) showed a much faster decrease in pH inside the skin biopsy compared to the ester of salicylic acid in preparation B. According to the literature the likewise highly lipophilic drug 2-hydroxyethyl-salicylic acid showed a high penetration rate into the skin, too. But contrary to azelaic acid the free acid is formed after the penetration of the 2-hydroxyethyl-derivative by esterases in the epidermis of the skin (18). Salicylic acid and ethyleneglycol were formed by an enzymatic breakdown. The delayed enzymatic formation of the free salicylic acid should explain the different pH gradients after application of preparations B and C as determined by means of ESR tomography.

CONCLUSIONS

The results of the current study indicate that drug penetration may lead to a change in pH values within the skin. The noninvasive technique of ESRI permits the direct and depth-specific measurement of pH values inside the skin. This is of particular importance with respect to the great number of acids and bases used epicutaneously. Thus ESRI is a powerful tool to evaluate species differences and to investigate the efficiency of penetration enhancers and drug carriers like liposomes and microemulsions.

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