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Cutaneous bioassay of salicylic acid as a keratolytic

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Abstract

Keratolytic efficacy of topical preparations containing salicylic acid was studied in humans utilizing adhesive tape stripping and quantifying SC removal by protein analysis. In combination with tape stripping, squamometry was used to evaluate the influence of salicylic acid on skin surface scaliness and desquamation. Furthermore, skin barrier perturbation and skin irritancy was recorded and related to the dermatopharmacological effect of the preparations. In contrast to squamometry, tape stripping combined with protein analysis was sensitive in detecting keratolytic effect of salicylic acid within hours of application. Importantly, whereas the pH of the preparations only minimally influenced efficacy, local dermatotoxicity was significantly increased at acidic pH. This indicates that the quest to increase the amount of free, non-dissociated SA is, in fact, counterproductive as the more acidic preparations resulted in skin irritation and barrier disruption.

Keywords: Hydroxy acid; Adhesive tape stripping; Skin physiology; Stratum corneum quantification; Protein assay; Dermatotoxicity

1. Introduction

Salicylic acid (SA, p$K_a \approx 3$ (Lide, 1999)), representing a pseudo β-hydroxy acid, is widely used as a peeling or keratolytic agent to treat callus, keratosis or warts (reviewed in Loden, 2000). SA is further applied in the treatment of acne, psoriasis and photoaging in various concentrations depending on the desired amount of keratolysis. Its keratolytic mechanism is not fully elucidated, but SAs dermatopharmacological effect may be related to its impact on the stratum corneum (SC) structure affecting intercorneocyte cohesion and desquamation. Although various methods including sequential adhesive tape stripping (Loden et al., 1995; Tsai et al., 1999) have been employed to assess the keratolytic effect of SA, they are limited or need further development

Abbreviations: SA, salicylic acid; SC, stratum corneum; TEWL, transepidermal water loss

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to accurately quantify its dermatopharmacological activity.

SC tape stripping is a minimally invasive technique to sequentially remove SC by repeated application of appropriate adhesive tapes (Pinkus, 1951). This technique can be used to investigate SC cohesion in vivo by quantifying the amount of SC removed (King et al., 1979). Today, weighing with precision balances is the most used method to determine the amount of SC removed on a tape strip. However, weighing may be biased by water absorption or desorption onto or from the tape (Marttin et al., 1996). Furthermore, after topical product application such as keratolytics, SC weighing is only reliable to some extent since the tape strips may not only contain SC but also applied vehicle and solute. As an alternative to weighing, a simple and detergent compatible colorimetric method based on a commercially available total protein assay was described (Dreher et al., 1998). Briefly, the assay is performed by immersing SC holding tapes in a sodium hydroxide solution in order to extract the soluble SC protein fraction. The SC protein containing solution is then neutralized with hydrochloric acid because the assay is not compatible with strong alkaline conditions. This quantification method makes it possible to determine accurately and reproducibly as little as a few microgram SC adhering to a single tape strip. Furthermore, the uptake of most product ingredients including water into the SC after topical product application does not interfere with this SC quantification method.

The study aimed to better understand the keratolytic effect of SA as a function of its formulation pH utilizing adhesive tape stripping and quantifying SC removal by the protein assay. In combination with tape stripping assessing SC cohesion progressively from the skin surface to the bottom layers, squamometry was used to evaluate the influence of SA on skin surface scaliness and desquamation. Furthermore, skin barrier perturbation and skin irritancy was recorded and related to the dermatopharmacological effect of the salicylic acid preparations.

2. Materials and methods

2.1. Human subjects

Six healthy adult subjects (three males and three females) aged between 20 and 40 years were enrolled. Subjects receiving topical medications, utilizing topical cosmetics at the test sites or those receiving anti-inflammatory medications were excluded, as well as volunteers with a history suggestive of allergy to adhesive tape. Subjects were required to acclimatize for 30 min prior to baseline bioengineering measurements. Subjects with hirsute forearms had the hair gently clipped at least 30 min prior to baseline readings. Written informed consent was obtained for all subjects and the study was approved by the Committee of Human Research, University of California, San Francisco.

2.2. Test preparations and treatment

The test preparations were (A) aqueous vehicle control of pH 7.4, (B) 2% SA aqueous solution of pH 3.3, (C) 2% SA aqueous solution of pH 6.95, and (D) 2% SA aqueous solution of pH 3.3. All preparations contained at least 10 wt.% specially denatured ethanol (SD alcohol 40). They further contained 5 wt.% propylene glycol. Preparation (D) contained in addition 1 wt.% menthol to provide a skin cooling effect after topical application. An untreated skin site (E) and an untreated, but occluded site (F) with an empty test chamber served as additional controls. The test preparations were coded. It was not possible to be blind to the open and occlusion controls. About 0.2 ml of the test preparations were pipeted onto a 25 mm plastic chamber (Hilltop Research, Ohio, IL) immediately prior to patch application and applied to the skin on premarked test sites secured with Scanpor® tape (Norgesplaster, Norway). Both volar forearms were utilized as the test site; one was randomly selected for sequential tape stripping and the other for squamometry. Test sites were randomized.

2.3. Skin irritation

Skin irritation was assessed visually according to the following scale: (0) normal skin and no erythema, (1) macular erythema, (2) erythema or edema, (3) vesiculation, and (4) ulceration or erosion. The skin was assessed prior to application of patches and 1 h after their removal. Skin color was measured with the Minolta Chromameter CR-300 (Minolta, Japan). The a^*-value was utilized as a measure of erythema (Fullerton et al., 1996). Each measurement was performed three times and the mean was recorded.

2.4. Tape stripping

An adhesive tape (D-Squame® 2.20 cm diameter, CuDerm, TX) was placed on the pre-marked test sites with forceps, applying a 10 kPa pressure uniformly over its entire area using a cylindrical weight system for 2 s. The tape was removed from the skin with forceps in a unidirectional manner at constant removal velocity for all sites (Löffler et al., 2004). 20 Tape strips were taken from each test site and were placed individually into a borosilicate glass vial (Fischerbrand scintillation vial, Philadelphia, PA) and refrigerated until further use.

2.5. Protein assay

The protein assay was performed according to the method described by Dreher et al. (1998). 1 ml of 1 M NaOH (Fisher Scientific, Pittsburgh, PA) was added to each vial containing a tape strip. Then, the vials were shaken for 2 h at room temperature in order to dissolve the SC protein fraction adhering to the tape. 1 ml HCl (Fisher Scientific) was added to the vials to neutralize the alkaline solution. Afterwards, the protein assay was performed using the Bio-Rad Detergent Compatible (DC) Protein Assay Kit® following the microassay procedure (Bio-Rad Laboratories, Hercules, CA). The kit included reagent A (an alkaline copper tartrate solution), reagent B (dilute Folin reagent) and reagent S (sodium dodecyl sulfate solution). Briefly, 200 μl of the neutralized SC sample solution was transferred into a 1.5 ml disposable cuvette (Bio-Rad Laboratories). Then, 100 μl of reagent A' (prepared by mixing 20 μl of reagent S to 1 ml of reagent A) and finally 800 μl of reagent B were added and mixed. After 30 min, absorbance at a wavelength of 750 nm was measured using a Hitachi U-2001 UV–vis Spectrophotometer (Hitachi Instruments, San Jose, CA).

2.6. Stratum corneum standard curve

Human cadaver skin from one donor was used to prepare SC sheets. The epidermis was obtained by heat separation (Kligman and Christophers, 1963) in water at 60 °C for 30 s and then placed overnight in 0.0001% (w/v) trypsin type III (Sigma, St. Louis, MO) in PBS (Sigma) at 37 °C. SC sheets were obtained after extensive rinsing with deionized water (prepared by Milli-Q; Millipore, Billerica, MA), drying under vacuum and finally quickly rinsing with ice-cold hexane (Fisher Scientific) in order to remove the superficial lipids adhering to the sheets. They were stored under vacuum at room temperature until use. In order to prepare a stock solution, a fixed mass of SC was dispersed in an appropriate volume of 1 M NaOH (Fisher Scientific) solution utilizing sonication and stirring at room temperature. From this stock solution, 10 serial dilutions were made with 1 M NaOH (Fisher Scientific) and assayed in an identical manner as described for the adhesive tapes to yield the SC mass standard curve. The amount of SC removed by tape stripping was calculated as follows: $\mu g\ SC\,ml^{-1}\ 1\,M\ NaOH = (OD_{750\,nm} - 0.0257)(0.0015)^{-1}$.

2.7. Squamometry

Squamometry was performed according to the method of Pierard et al. (1992). One tape disc (D-Squame, 22 mm) was placed to the skin at each test site followed by application of a 10 kPa pressure for 5 s using a cylindrical weight system. Thereafter, the tape was gently removed utilizing forceps and each tape disc was stained with one to two drops of a solution of toluidine blue and basic fuchsin (Polychrome Multiple Stain; Delasco, Council Bluffs, IA) for 30 s. The tape discs were rinsed with deionized water, dried on a slide warmer and finally mounted onto a glass slide. The stained tape discs were evaluated by colorimetry using a Minolta Chroma Meter CR 300 (Minolta, Osaka, Japan). Three measurements in the Lab-space were performed to calculate the squamometry index $C^* = (a^{*2} + b^{*2})^{1/2}$ as well as the colorimetric index of mildness $CIM = L^* - C^*$. In addition, a microscopic evaluation of the stained discs was performed at 100-times magnification using the 5-point scales for intercorneocyte cohesion and cell staining. The scales for intercorneocycte cohesion were: (0) large sheet, (1) large clusters and few isolated cells, (2) small clusters and many isolated cells, (3) clusters in disruption and most cells isolated, and (4) all cells isolated and many cases of lysis. The scales for amount and distribution of dye within the cells were: (0) no staining, (1) staining between cells or slight staining, (3) moderate, but uniform amount of dye in cells, and (4) important staining in all cells, often with grains.

2.8. Transepidermal water loss

Transepidermal water loss (TEWL) represents a measure for skin barrier integrity and was measured utilizing an evaporimeter (Tewameter® TM210; Courage-Khazaka, Germany; Academ, Menlo Park, CA). TEWL readings were taken before treatment (baseline value) and then 1 h after removal of the occlusive patches to allow for deconvolution from occlusive super-hydration. TEWL was also recorded after 10 and 20 tape strips at each test and control site. At least 2 min were allowed to elapse between removing the preceding tape and measuring TEWL to allow for redistribution of water within the stratum corneum. Room temperature and humidity were monitored and ranged between 19 and 21 °C and between 50 and 60% relative humidity during the study.

2.9. Data analysis and statistics

Statistical analysis was performed utilizing SigmaStat 2.03 (SPSS Inc., Chicago, IL). For non-parametric data, such as clinical scoring, data was analyzed utilizing the analysis of variance (ANOVA) for ranks combined with Dunn's pair-wise comparison. Parametric data such as a^*-values were analyzed with the repeated measures one-way ANOVA combined with the Tukey or Bonferroni pair-wise comparison. Statistical significance was accepted when $p < 0.05$. TEWL was analyzed utilizing the one-way repeated measures analysis of variance with S–N–K test for multiple pair-wise comparisons.

3. Results

3.1. Skin irritation

As assessed visually (Fig. 1), neither vehicle (A), nor occlusion (F) induced erythema or edema in any of the subjects; the clinical scores were 0. The salicylic acid pH 3.3 solution (B) induced confluent macular erythema in three subjects as well as edema in three subjects, giving a median score of 1.5. The salicylic acid pH 6.95 solution (C) was less irritating with a median score of 0.25. The salicylic acid pH 3.3 solution containing menthol (D) irritated all subjects with a median score of 1. The occlusion control test site and the

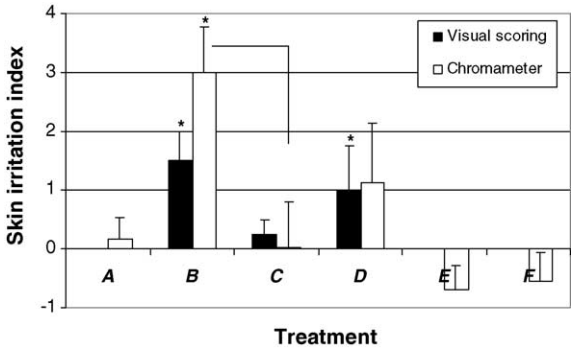

Fig. 1. Skin irritation, evaluated visually (median and 75th percentile of irritation score) and by Chromameter (mean ± S.E.M. of Δa^*-value) after product application, as a function of treatment with preparation A to F (see 'Section 2' for composition). Significant differences between SA-treatments and baseline as well as between treatments and vehicle (A), occlusion (F) or, respectively, untreated control site (E) are indicated (*). Significant differences between SA-containing preparations are further indicated.

untreated, unoccluded test site both yielded a median of 0. Preparations B and D were significantly (ANOVA on ranks, S–N–K pair-wise comparison) more irritating than the vehicle (A), the occluded control (F) and the untreated control site (E). The differences between preparations C and B, or respectively, D did not reach significance. Changes in chromameter a^*-values were expressed as differences (Δa^*) between baseline values and after treatment (Fig. 1). For some subjects, the a^*-value decreased post-treatment, even after allowing 1 h for the skin to recover after occlusion. Only treatment with salicylic acid pH 3.3 solution (B) resulted in a significantly increased a^*-value as compared to baseline measurement. Otherwise, treatment with preparation D resulted in a slightly increased, but not statistically significant higher a^*-value as compared to baseline measurement ($p = 0.06$). When comparing Δa^*-values between treatments, preparation B resulted in a significantly (ANOVA on ranks, S–N–K pair-wise comparison) greater a^* than observed after treatment with vehicle (A), the salicylic acid pH 6.95 solution (C) as well as for the occluded control (F) and the untreated control site (E), respectively.

3.2. Transepidermal water loss (TEWL)

TEWL increased significantly after treatment with the salicylic acid pH 3.3 solution (B) and the

salicylic acid pH 3.3 solution containing menthol (D) as compared to treatment with the salicylic acid pH 6.95 solution (C), the vehicle (A) as well as the occluded (F) and the untreated control site (E), respectively (Fig. 2). The increase in TEWL after treatment was $2.3 \pm 1.1\,gm^{-2}\,h^{-1}$ (mean ± S.D.) for preparation A, $5.3 \pm 1.9\,gm^{-2}\,h^{-1}$ for preparation B, $2.4 \pm 1.6\,gm^{-2}\,h^{-1}$ for preparation C, and $5.1 \pm 3.1\,gm^{-2}\,h^{-1}$ for preparation D. Following 10 or 20 tape strips, skin site treated with either the salicylic acid pH 3.3 solution (B) or the salicylic acid pH 3.3 solution containing menthol (D) showed a significantly increased TEWL as compared to the vehicle (A), the occluded (E) as well as the untreated control (F) sites, respectively. Furthermore, the increase in TEWL was significantly greater after 10 and 20 tape strips at the site where the salicylic acid pH 3.3 solution (B) was applied as compared to the salicylic acid pH 6.95 solution (C). Whereas the increase in TEWL was significantly greater for the salicylic acid pH 3.3 solution containing menthol (D) as compared to preparation C after 10 tape strips, no such difference was found after 20 strips.

3.3. Squamometry

Microscopic analysis of the tapes did not reveal significant differences in the intercorneocyte cohesion or cell staining (no data shown). Further, there was no significant difference in the squamometry index (C^*) as well as the colorimetric index of mildness (CIM) between the treatments (no data shown).

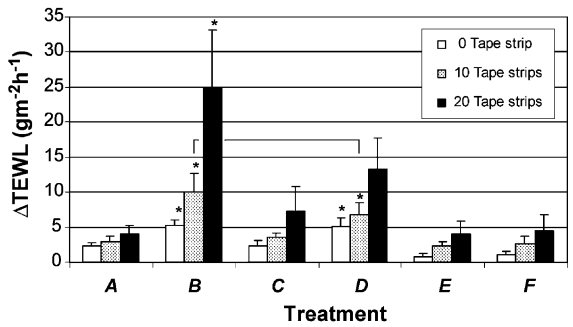

Fig. 2. Skin barrier integrity as assessed by TEWL after treatment and following 10 and 20 tape strips as a function of treatment with preparations A to F (see 'Section 2' for composition). Differences in TEWL (ΔTEWL, mean ± S.E.M.) between treatment and corresponding baseline value are given. Significant differences between preparations (B) or (D) and preparation (C), vehicle (A), occlusion (F) or, respectively, untreated control site (E) are indicated (*). Significant differences between preparations (B) and (D) are further indicated.

3.4. SC mass removed after tape stripping

The cumulative mass sum of SC removed by sequential tape stripping after each treatment is shown in Fig. 3. After 10 and 20 tape strips, significantly (one-way ANOVA for repeated measures, with S–N–K pair-wise comparisons) more SC was removed by tapes stripping in test sites treated with salicylic acid preparations (C) and (D) as compared to the vehicle (A) and with the open (F) and occluded control (E) sites. Each of the effective treatments (C, D) stripped more than double the amount of SC than the vehicle (A) or the control sites. Treatment with the salicylic acid pH 3.3 solution (B) resulted also in an increased amount of SC removed after 10 and 20 tape strips as compared to vehicle (A) and the other control sites (E, F). However, this difference was only significant after 20 tape strips. Treatments with the salicylic acid pH 6.95 solution (C) and the salicylic acid pH 3.3 solution containing menthol (D) removed significantly more SC in the first 10 strips as compared to treatment with the salicylic acid pH 3.3 solution (B). After 20 strips, however, similar and not statistically different SC amounts were removed for all preparations containing salicylic acid (B, C and D). Moreover, there was no statistical

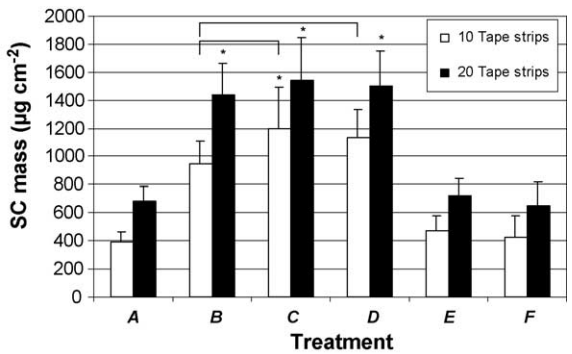

Fig. 3. Mass ($\mu g\,cm^{-2}$, mean ± S.E.M.) of SC removed after 10 and 20 tape strips as a function of treatment with preparations A to F (see 'Section 2' for composition). Significant differences between SA-treatments and vehicle (A), occlusion (F) or, respectively, untreated control site (E) are indicated (*). Significant differences between SA-containing preparations are further indicated.

difference in the mass of SC stripped between any of the control sites including vehicle (A) and occlusion control (E).

4. Discussion

The study describes an accurate and easy procedure to assess the efficacy of keratolytics and their formulation dependency in humans in vivo. Utilizing the protein assay to accurately quantify SC removal after tape stripping in combination with cutaneous biometrics represents a significant progress in the quantitative assessment and evaluation for keratolytic and desquamative therapies.

Previously, varieties of simple to more complex methods have been employed to determine the efficacy of SA in humans. Davies and Marks (Davies and Marks, 1976) were unable to determine histological differences between skin treated with SA compared to vehicle on regular light microscopy but found differences between the two sites on scanning electron microscopy. Without any means to objectively quantify the degree of desquamation, the scanning electron microscope images led the authors conclude that SA was likely to dissolve intracellular cement. Another method to elucidate the effect of 6% SA (in 70% ethanol) on normal human skin utilized forced desquamation cell counts (Roberts et al., 1980). An increased number of corneocytes shed by forced desquamation in the SA treated skin was reported in comparison to control sites (70% ethanol) after 8 h application; though no statistical analysis was presented. In the 10-day study, the SC was stained with two dyes (sliver nitrate, dansyl chloride) in order to quantify corneocyte loss by determining the change in stain density with time. This method did not reveal a statistical difference between the control and treated skin for either stain, although it was noted that the rate of stain loss for silver nitrate was greater in SA treated skin as compared to vehicle control. However, the 10-day study did clearly demonstrate, a significantly reduced number of SC cell layers in SA treated sites compared to vehicle treated sites as assessed by histology. In another study employing also silver nitrate to quantify the keratolytic activity of SA, Nook (1987) described the method as being able to only detect changes after a prolonged study period following product application, but that large standard deviations led to difficult product comparisons. Summarizing, these studies suggest that visual assessment of the SAs keratolytic activity, whether by microscopy or by observing staining patterns on the skin surface, may take several days of application for detecting an effect.

In comparison to these studies, the present study identified clear statistical differences in the amount of SC removed already after a 6 h treatment with SA on the volar forearm. This suggests that sequential tape stripping is sufficiently sensitive to detect early desquamative effects confirming the results of a comparable study by Lodén (2% SA dissolved in a hydro-alcoholic solution) (Loden et al., 1995), where SAs keratolytic effect could be demonstrated after 6 h, but not after 3 h of occlusive application. Lodén and coworkers assessed SC removal semi-quantitatively by measuring the light transmission through the tape, whereas SC removal was determined with the protein assay in the present study. In contrast to weighing, and eventually also to the former quantification method, the protein assay enables to quantitatively determine the SC mass on a tape independent of the SCs unbound water content. This factor is particularly relevant when the preparations are studied under occlusive conditions as done in these studies.

The squamometry data helped to further elucidate the mode of action of SA as a desquamative active ingredient. We anticipated that keratolytic treated sites would demonstrate a more scattered arrangement of corneocytes on the tape as compared to vehicle treated sites which would demonstrate a more intact corneocytes sheet on the tape surface. In fact, there was no significant difference in the arrangement of corneocytes on the tape, all sites demonstrated a relatively intact sheet independent of treatment. The sensitivity of this method seems therefore not sufficient to detect a keratolytic effect after a short treatment period under occlusion. As shown in a separate study under in-use conditions, squamometry allowed detecting an altered intercorneocyte cohesion only after a prolonged and repetitive application of 2 weeks and more (Pierard-Franchimont et al., 1998). The authors of this study indicated that squamometry represents not a direct measure for the desquaming effect of SA and other keratolytics since lost corneocytes are not accounted by this method. The observation that the keratolytic effect of SA was not detectable by squamometry after

a short-term, occlusive application appears to be consistent with the findings of previous studies reporting difficulties to observe a keratolytic effect of SA using dyes such as silver nitrate (Roberts et al., 1980; Nook, 1987).

As for most solutes, SA penetrates across skin better in its neutral form compared to its ionized form (Marcus et al., 1970; Leveque et al., 2004). On the other hand, the skin permeability of salicylate may be facilitated depending on the presence and properties of counter-ions (Megwa et al., 2000a, 2000b). Since SC is the target tissue for keratolytic activity, formulations optimized to deliver SA in this skin compartment presumably show most therapeutic efficacy. As a consequence, a SA containing formulation designed for optimal systemic absorption may not necessarily show highest keratolytic efficacy. The fact that preparation C (pH 6.95), where SA is mostly present as salicylate, showed a comparable keratolytic activity than preparations B and D (pH 3.3, approximately 65% present as salicylate (without taking into account the influence of the formulation ingredients on the dissociation constant of SA)) corroborates this hypothesis, which was already postulated before by others (Neubert et al., 1990). This suggests that the uptake of SA into the SC was sufficient from the neutral preparation C to produce a pronounced keratolytic effect.

The skin water barrier integrity in relation to SA treatment was further studied by TEWL. Different formulations of 2% SA affected the skin barrier differently, following a pattern related to their pH. However, TEWL represented no predictive measure for a keratolytic effect after SA treatment. TEWL increased significantly after both treatments with acidic pH preparations (B and D). However, for treatment C, which was similarly effective as a keratolytic preparation, there was no significant rise in TEWL as compared to baseline. This suggests that SA may significantly decrease SC cohesion by only minimally disrupting skin barrier to water diffusion. Since corneodesmosomes are believed to be the major component providing SC cohesion (Egelrud et al., 1988; Chapman et al., 1991), our results indicate that SA mainly affects their structure and seems not significantly to perturb the SC lipid composition and organization, which is mainly responsible for SCs excellent barrier properties. However, the mechanism of SAs interaction with corneodesmosomes after a single and short term application remains unknown and needs further investigation.

In addition to monitoring skin water barrier function by TEWL, we also documented skin erythema after SA application. At least for SA, we demonstrated that acute skin irritation must not be a necessary side effect for a keratolytic effect. This observation contrasts studies alluding keratolytic efficacy of alpha-hydroxy acids with some dermatotoxicity (reviewed in Lewis, 2001). Hence, the preparation containing 2% salicylic acid at neutral pH (C), induced only a slight TEWL change, was minimally irritant, whilst retaining its ability to be as keratolytic as similar preparations at acidic pH (B and D). This demonstrates that effective desquamation can be achieved in the absence of any clinical signs of cutaneous irritation. These findings suggest that the keratolytic effect of SA is not simply the result of a non-specific skin irritant effect, but is rather a real pharmacological effect.

Concluding, using tape stripping in combination with a protein assay to accurately quantify SC removal substantiated earlier reports that SA acts as a significant keratolytic. This experimental model appeared sensitive in detecting efficacy of SA preparations within hours of application. Importantly, whereas the pH of the SA containing preparation only minimally influenced the keratolytic efficacy, the dermatotoxicity was significantly increased at acidic pH instead. In light of this, our data indicates that the quest to increase the amount of free, non-dissociated SA is, in fact, counterproductive as the more acidic preparations resulted in skin irritation and barrier disruption. These properties are especially undesirable as keratolytic products containing hydroxy acids are frequently used on the face to improve the condition of lesional skin and enhance cosmetic appearance. Hence, a 19th century drug has allowed new insights in the 21st century; much still remains to be understood.

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