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Does lactobionic acid affect the colloidal structure and skin moisturizing potential of the alkyl polyglucoside-based emulsion systems?

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Moisturizing creams are the most prescribed products in dermatology, essential in maintaining healthy skin as well as in the topical treatment of some diseases. The irritation potential of commonly used emulsifiers and moisturizing ingredients, but also their mutual interactions, could affect the functionality and safety of those dermopharmaceutics. The aim of this study was to promote moisturizing alkyl polyglucoside (APG)-based emulsion as vehicle for lactobionic acid (LA), advantageous representative of the alphahydroxyacids (AHAs)-multifunctional moisturizers, assessing the safety for use (*in vitro* acute skin irritation test using cytotoxicity assay compared with *in vivo* data obtained using skin bioengineering methods) and *in vivo* moisturizing capacity (bioengineering of the skin). In order to investigate possible interactions between APG mild natural emulsifier-based emulsion and LA, a deeper insight into the colloidal structure of the placebo and the emulsion with LA was given using polarization and transmission electron microscopy, rheology, thermal and texture analysis. This study showed that APG-based emulsions could be promoted as safe cosmetic/dermopharmaceutical vehicles and carriers for extremely acidic and hygroscopic AHA class of actives (specifically LA); prospective safety for human use of both APG and LA with the correlation between *in vivo* and *in vitro* findings was shown. However, it was revealed that LA strongly influenced the colloidal structure of the emulsion based on APGs and promoted the formation of lamellar structures which reflects onto the mode of water distribution within the cream. The advantageous skin hydrating potential of LA-containing emulsion *vs.* placebo was unlikely to be achieved, pointing that emulsions stabilized by lamellar liquid crystalline structures probably are not satisfying carriers for highly hygroscopic actives in order to reach the full moisturizing potential. Safe and effective use on dry skin is presumed.

1. Introduction

Creams are a common type of delivery systems in moisturizers category and often prescribed dermatological products mainly intended for the treatment of dermatoses accompanied by the clinical and subjective impressions of skin dryness (Loden 2005). In the group of moisturizers, emulsions stabilized by lamellar liquid crystals (LLC) are intensively investigated as carriers for various dermopharmaceutics/dermocosmetics, considering their extensive similarity to the systems found in living organism (e.g. *stratum corneum* (SC) lipid matrix), significant solubilisation capacity for lipophilic and hydrophilic substances and particularly the potential for sustained skin hydration (Junginger 1997; Savic et al. 2009). In fact, moisturizers are essential in enabling the skin to stay intact as well as in the topical treatment of some diseases such are psoriasis, ichtyosis, atopic dermatitis (Fluhr et al. 2008), thus they should exhibit good tolerability. That requires the use of well tolerated surfactants and actives, taking into account possible interactions between those ingredients.

It is a well known fact that, although irritant for the skin, alphahydroxyacids (AHAs) are among the most effective and widely used multifunctional active substances with a moisturizing effect (Yu and Van Scott 2004). The newer, much milder dermopharmaceutic/dermocosmetic actives are polyhydroxy acids (PHAs) (Green et al. 2009; Yu and Van Scott 2004) that include as a typical representative the lactobionic acid (LA), a highly hygroscopic "*superacid*" with multiple hydroxyl groups (Briden and Green 2005), which therefore presents a formulation challenge, particularly when incorporated into emulsion systems. LA is also known to possess an antioxidant effect (Briden and Green 2005). Traditionally, emulsion carriers for acidic actives in dermopharmacy are often based on acid-stable nonionic polyethylene glycol (PEG)-emulsifiers (e.g. etoxylated fatty alcohols and fatty amphiphiles). Although showing a structure of lamellar liquid crystals (LLC) and/or lamellar gel-crystalline type, these emulsions could induce erythema in exposed healthy skin and its barrier impairment (Bárány et al. 2000). In addition, it was presumed that AHAs could act as hydrotropes and cause disorder of LLC structures (Al-Bawab and Friberg 2004). Therefore, it could be of interest to develop a stable, aesthetically pleasing and non-irritant vehicle stabilized by skin-friendly LLC structures containing the AHA-type of skin moisturizing actives.

The above statements point to the possible use of natural, biodegradable nonionic sugar-based emulsifiers of the alkyl polyglucoside (APG) type, which also form lyotropic mesophases and are mild to the skin (Stubenrauch 2001; Savic et al. 2009) as effective excipients useful in the formulation of vehicles for both dermatologic drugs and cosmetic actives. It was presumed that a large number of hydroxyl groups present in APG emulsifiers may contribute to the overall skin hydration potential of APG-based emulsion systems (Savic et al. 2009). Recently, we have reported the detailed *in vitro/in vivo* characterization of an APG–based emulsion as a carrier for LA. Namely, an excellent safety profile and skin hydration potential of the APG-based emulsion containing 6% of LA was shown (Tasic-Kostov et al. 2010). Although the used C12/14 APG emulsifier has been proved to be an acceptable stabilizer for an emulsion system loaded with an acidic active (LA), the mentioned study failed to show the synergism between LA and the APG-based emulsion system in skin hydration potential. A possible reason for the absence of the expected synergism could be the interaction between LA and the carrier, which may reduce the effect of skin hydration.

Consequently, the current study consisted of two parts.

The aim of the first part (part I) of the study was to give a deeper insight into the colloidal structure of the APG-mixed emulsifier (INCI/Cetearyl alcohol and coco glucoside) stabilized emulsion system (placebo and the emulsion with 6% of LA). The polarization and freeze-fracture transmission electron microscopy (TEM), differential scanning calorimetry (DSC), thermogravimetric analysis (TG), texture analysis and rheology measurements were employed. We particularly considered the possible interactions between LA and the APG emulsifier within the system.

Afterwards, the second part (part II) of the study aimed to assess the emulsion based on APGs containing a higher percent of LA (10%). We were interested to find out whether the increase of LA content affects physical stability (assessed by rheology, pH and conductivity measurements and polarization microscopy), *in vitro/in vivo* safety-irritation potential and moisturizing capacity of the emulsion vehicle. Certain aspects of safety profile were evaluated *in vitro* using an alternative method for acute skin irritation test (a cytotoxicity assay) (Vinardell and Mitjans 2008), while in the 24h-*in vivo* study under occlusion the following human skin parameters were measured: SC hydration (SCH), transepidermal water loss (TEWL), and skin erythema index (EI) (Bárány 2000). In addition, we checked the *in vivo* moisturizing efficacy of this sample in both long-term (24 d) and short-term (3 h) studies measuring SCH.

Overall, the aim of this study was to assess whether the vehicle based on natural APG emulsifier with the addition of LA, both claimed to provide an excellent skin hydration, can give a physically stable moisturizing emulsion with improved efficacy and acceptable safety excluding at the same time the drawbacks typical for their old-generation representatives.

2. Investigations, results and discussion

2.1. Part I of the study

We have formulated and tested a multicomponent emulsion (placebo, marked as M82) based on C12/14 APG (Montanov®82) emulsifier and the "active" creams which were of the same composition as placebo, with the addition of LA, labeled as: M82L1N-sample with 6% (w/w) LA; M82L1 – the same as M82L1N with pH value adjusted to comply with the required values (3.5–4.5).

Having in mind our previous results (Tasic-Kostov et al. 2010), the current study has considered a presumable interaction between an acidic active with strong humectant effect (LA) and the colloidal structure of C12/14 APG-based vehicle. It was of interest to ascertain the microstructure of both placebo (M82) and LA-containing (M82L1 and M82L1N) samples; the physical stability of those samples was previously shown (Tasic-Kostov et al. 2011).

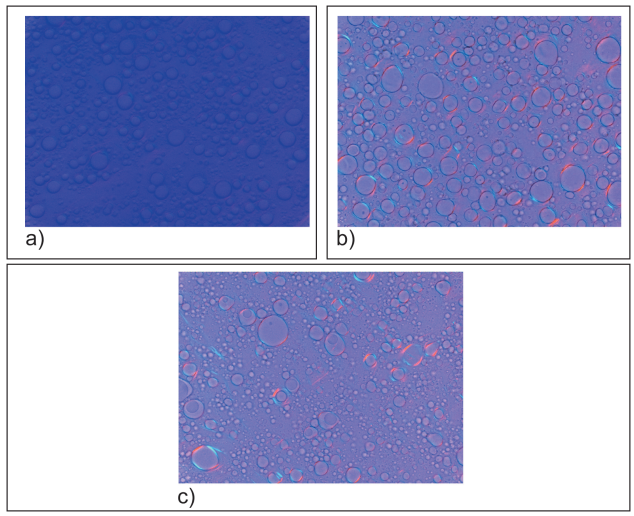

Fig. 1: Polarization micrographs of the samples a) M82; b) M82L1; c) M82L2; magnification 400x

Polarization microscopy of the sample M82 (Fig. 1a) has revealed only poorly developed stripes of lamellar mesophases between the oil droplets, while TEM micrograph of this sample shows few typical features which indicate the existence of anisotropy, probably originating from the lamellar gel phase (Fig. 2a). On the other hand, the addition of LA influenced the clear anisotropy of the active samples M82L1 (Fig. 1b) and M82L1N (similar as in M82L1, data not shown), appearing as droplets uniformly dispersed into the continuous phase with small stripes of lamellar mesophase surrounding them (distorted Maltese crosses, the so called "onion rings"), pointing to well developed lyotropic interaction of the lamellar type. The TEM micrographs corresponded well to the polarization ones, confirming the existence of lamellar gel phase at the edges of the oil droplet in the sample M82L1 (Fig. 2b). Therefore, a significant impact of the LA and its salts incorporation (after the neutralization) into the APG-based vehicle could be indicated by these findings, which are additionally substantiated by the oscillatory rheological measurements. Although viscoelastic rheological behaviour, typical of the lamellar structures has been detected in all test samples, clear differences were shown depending on the LA incorporation.

The results of continual and viscoelastic rheological testing are given in Table 1 and Fig. 3. The storage moduli (G') was higher

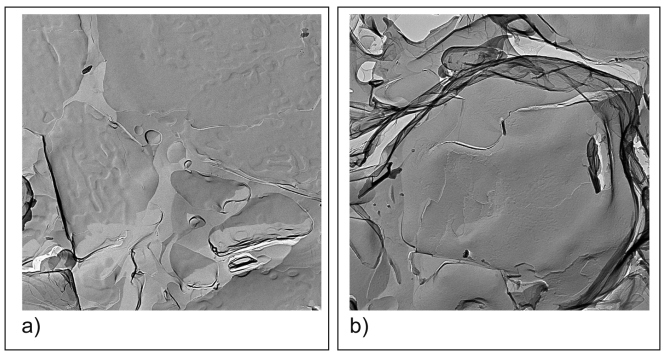

Fig. 2: TEM micrographs of the samples a) M82; b) M82L1; magnification 4000x

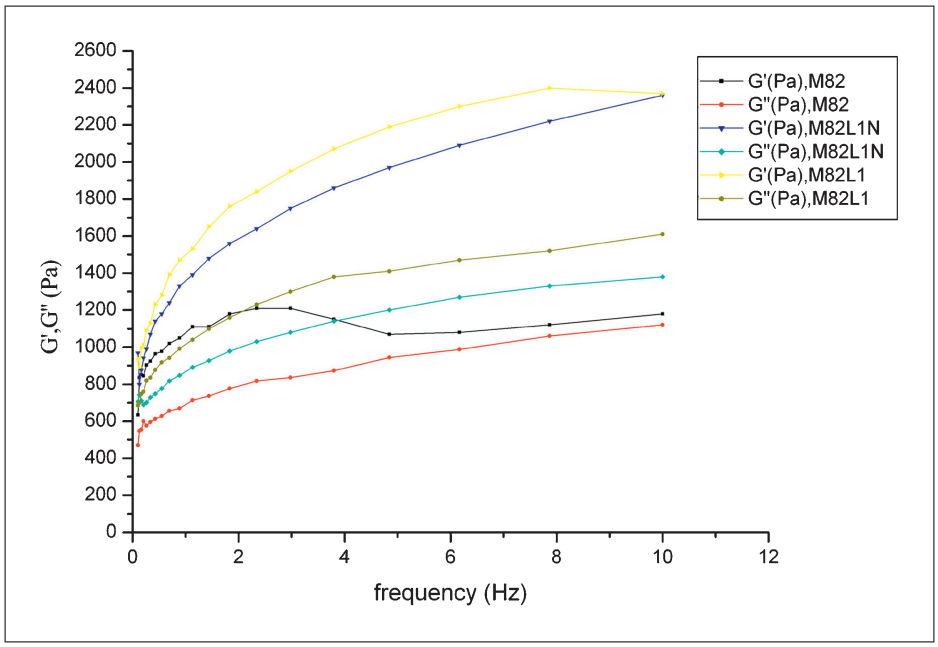

Fig. 3: Viscoelastic behaviour of placebo and the samples with 6% LA

than the loss moduli (G") for all the investigated samples; sample M82 had the lowest G'/G" ratio with the lowest G', pointing to the stabilization of this sample by α-crystalline gel phase predominantly. With the increase of applied frequency, G' for M82 exhibited great decline which is in line with microscopy findings on a probably unpronounced or extremely weakly pronounced LLC phase. The LA-containing samples exhibited much higher elastic (storage) moduli compared to the placebo, with the marked prevalence of the elastic over the viscous component. Such a finding could point to a certain lamellar liquid crystalline structures promotion by the active substance, LA and its salts (formed after neutralization); this effect was particularly pronounced in neutralized sample M82L1. Furthermore, G' for both active samples has shown small variations with the change of frequency, which indicates a favourable rheological behaviour-good resistance when exposed to stress conditions (Korhonen et al. 2005).

However, the results of oscillatory measurements were not in full alignment with continuous rheology which has revealed higher viscosity values in M82L1N (η_{max}, Table 1), but correlated well with the texture analysis (penetration studies) findings. Table 2 shows a range of textural parameters after 7 days of storage. M82L1 has revealed higher values of both firmness and stickiness, which was in correlation with the values for viscous modulus obtained for that sample.

pH and conductivity values of all tested samples (both parts of the study) are shown in Table 3. pH of the placebo was as recommended for products intended for the skin administration, and indicated a mild nature of the used APG surfactant, while pH of the active samples depended on neutralization degree. In contrast to the samples stabilized with C16/18 APG mixed emulsifier (Savic et al. 2009), conductivity value of the sample

Table 1: Flow and oscillatory (at frequency of 1 Hz) parameters of the samples

Sample	η_{min} (Pas) 4 s^{-1}	η_{max} (Pas) D 200 s^{-1}	G′ (Pa)	G″ (Pa)
M82	0,346	7,12	1110	713
M82L1N	0,412	8,00	1390	891
M82L1	0,339	5,98	1530	1040

M82 (over 50 μs/cm, Table 3) indicated an o/w emulsion system, the finding potentially explained (Tasic-Kostov et al. 2011) by the higher molecular polarity of coco glucoside (C12/14) mixed emulsifier. It is well established that the conductivity of the multiphase emulsion systems formed by APG-based mixed emulsifier corresponds to the fraction of free/bulk water and the concentration of the ions in it (Korhonen et al. 2005). LA samples had somewhat higher conductivity values, which are reasonable to presume; furthermore, much higher values were expected regarding the LA electrolytic nature. Conductivity values were not ranked similarly to apparent viscosities (Table 1) of the samples M82 and M82L1N, although it is expected for multiphase emulsions (Korhonen et al. 2005). It could be speculated that in the active samples the ionic nature of LA and its salts after neutralization imparts the high conductivity values, while the impact of the free fraction of water on the total conductivity is insignificant, indicating that the large quantity of water is bound between the formed structures. That is in line with the data on the microstructure of the systems obtained using TEM and polarization micrographs (Figs. 1 and 2).

Thermoanalytical examinations, DSC and TG, served to differentiate between variously bound water in the investigated creams due to a sufficiently large difference in the free energies between different types of water within the system (Csizmazia et al. 2010; Fairhust et al. 1998). The obtained DSC results are presented in Fig. 4. The placebo sample M82 had only one marked peak at 61.5 °C which, regarding relatively high total enthalpy of 7.42 mJ/mg probably corresponds to the melting of the lamellar gel phase and the evaporation of incorporated water (Nesseem 2008). The active sample M82L1 had two peaks: at 54.5 °C and a poorly marked one at 66.8 °C, which could be explained by the water redistribution inside the system. The shift of the first peak toward lower temperatures alongside the decrease in the total enthalpy could indicate the movement of the part of incorporated water to the loosely bound/free water while the appearance of the second peak (with lower enthalpy, 1 mJ/mg) at higher temperature could imply the shift toward the lamellar liquid crystalline structures, characterized by higher mobility and lower enthalpy compared to ordered crystalline structures (Fairhust et al. 1998). This is in accordance with the previous findings from this study, and it also implies the formation of the layered lamellar liquid crystalline structures formed at oil

Table 2: Textural parameters of the samples after 90 days of storage

Sample	Maximal force (g)	Maximal area (g·sec)	Minimal force (g)	Minimal area (g·sec)	Travel (mm)
	Firmness	Work of penetration	Stickiness	Work of adhesion	Cohesiveness
M82	19.44	155.02	9.61	100.46	23.53
M82L1N	18.21	145.43	9.38	90.87	21.29
M82L1	42.69	326.85	19.67	209.67	23.11

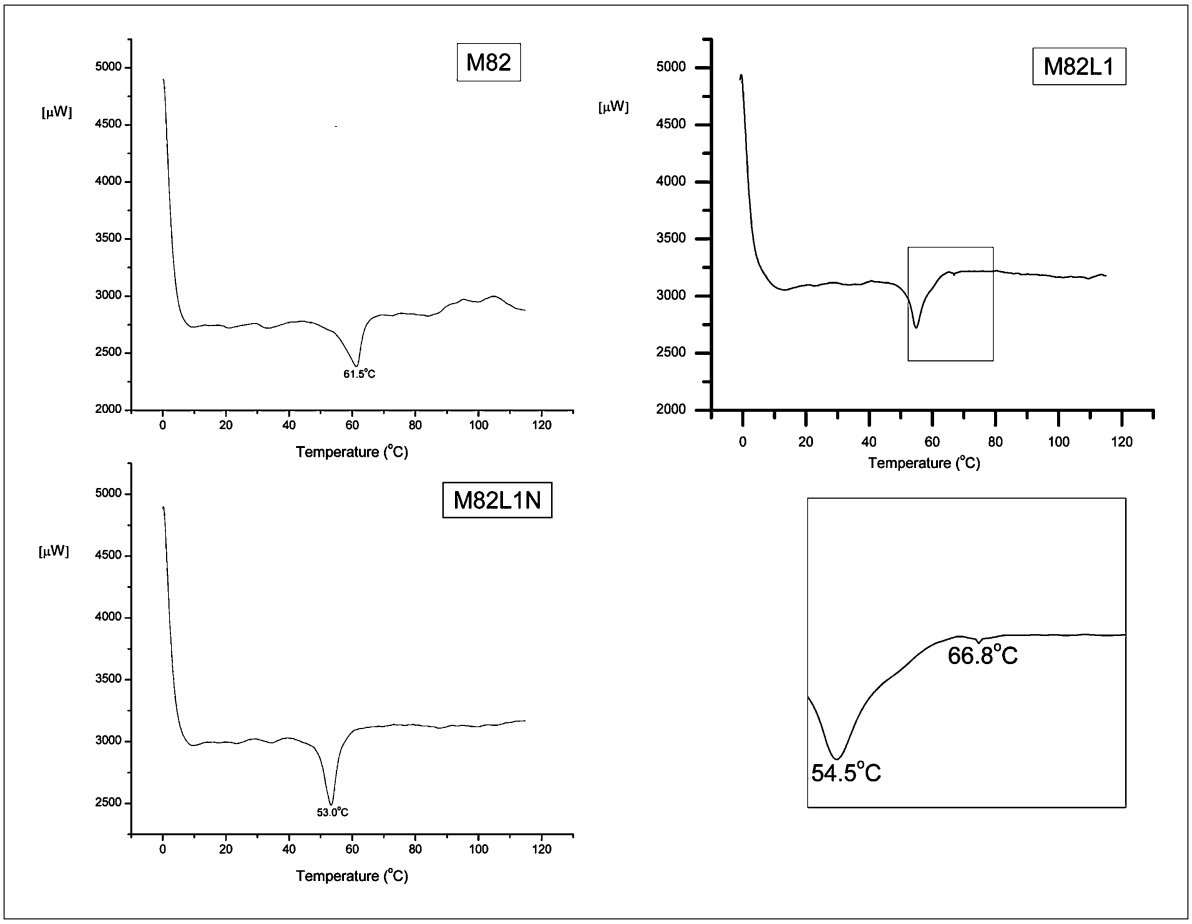

Fig. 4: DSC profiles of the samples M82, M82L1, M82L1N

droplets border in the presence of LA, while in placebo sample the complex crystalline gel dominates within the continual phase.

In addition to DSC measurements, TG and derivative TG (DTG) curves (Table 4, the results are given as means ± standard deviation) representing the water loss from the sample as a function of temperature, were used for the investigation of water distribution

Table 3: pH and conductivity values after 7, and 60* days of storage

Sample	pH	Cond. (µS/cm)
M82	6.54	72.80
M82L1N	2.48	101.00
M82L1	3.80	188.70
M82L2N	3.80	99.00
	3.50*	118.70*
M82L2	3.80	89.09
	3.55*	122.00*

mode within the creams. Such findings may correlate with the *in vivo* evaluated moisturizing potential of the semisolid topical preparations (Csizmazia et al. 2010). The obtained results have not revealed a difference in the level of structuration between placebo and active samples. Namely, regarding the other findings from part I, it was expected that the active samples had a higher fraction of water which may evaporate between 70 °C and 100 °C, corresponding to the fraction of interlamellar water, potentially fixed between the lamellae of the liquid crystalline phase ("depot" water), formed in the LA-containing sample; the decreased fraction of water that evaporates between 50 °C and 70 °C, and mostly corresponds to the water mechanically entrapped within the lipophilic gel phase (Junginger 1997; Savic et al. 2009) was also expected. Our results (Table 4) showed that the samples had almost equal amounts of bulk (evaporates below 50 °C) and incorporated water while the highest amount of water was lost between 50 °C and 70 °C in all samples. These results could probably be explained in the light of the unexpected *in vivo* findings from our previous study (Tasic-Kostov et al. 2010). Namely, LA, a highly hygroscopic substance with pronounced water-binding capacity, forms a gel matrix when its aqueous

Table 4: Percentage of total and partial weight losses over the specified temperature ranges represented as mean ± SD

Sample	Total weight loss (%)	25–50 °C (%)		50–70 °C (%)		70–100 °C (%)		Residual mass (%)
M82	68.19 ± 1.89	14.14 ± 0.29	19.49 ± 0.66*	28.77 ± 1.09	39.69 ± 1.02*	25.28 ± 0.92	34.87 ± 0.58*	31.71 ± 1.11
M82L1	57.91 ± 1.74	13.68 ± 0.98	20.72 ± 0.78*	23.00 ± 0.87	34.86 ± 0.80*	21.23 ± 1.01	32.16 ± 0.91*	41.98 ± 0.67
M82L1N	61.77 ± 1.92	15.23 ± 0.83	22.90 ± 0.93*	24.08 ± 1.06	36.21 ± 1.01*	22.46 ± 1.21	33.78 ± 0.99*	38.13 ± 1.20

* water loss relative to the water content per sample

solution is evaporated at room temperature; a certain amount of water is strongly bound and does not evaporate (Green et al. 2009). It could be speculated that LA also binds and withholds a large part of the water within the cream, particularly the fraction fixed between the lamellae of the liquid crystalline phase, affecting in that way the TG results and limiting the full moisturising potential of the active sample. Similar reports have been made for urea, which is also a highly hygroscopic substance, when incorporated into the APG-based emulsion. In that study (Savic et al. 2004) the significant synergism in the moisturization effect between the vehicle and the active was observed only in dry skin and was explained by different responses of normal and dry skin to the urea as the hydrating active.

The first part of the study clearly implies that LA, a highly acidic and hygroscopic topical active belonging to the advanced class of well-known AHAs, interacts with the colloidal structure of the emulsion based on APG-type emulsifier cetearyl alcohol and coco glucoside. It could be speculated that LA and its salts (formed after neutralization) here promote the formation of lamellar liquid crystalline structures; the part of hydrosoluble LA its salts (concentration of 6%), bound with water molecules by hydrogen bonds is probably incorporated into interlamellar spaces of the formed lamellar liquid crystalline structures placed around dispersed oil droplets, whereas the rest is dissolved in the bulk water. The obtained results were opposed to the expected ones. Namely, even though this study shows that complex structures with strong water binding capacity which can deliver the differently bound water in several steps to the skin (Csizmazia et al. 2010) are formed in LA-containing emulsion, our previous study (Tasic-Kostov et al. 2010) has not shown better skin hydrating capacity of this sample over placebo.

2.2. Part II of the study

In the second part of the study we aimed to investigate the effects of the M82 emulsion with 10% LA in order to find out whether this APG-based vehicle could be declared as appropriate for incorporation of high percents of LA. We investigated if the increased LA content would affect physical stability, safety profile and moisturizing capacity of the emulsion vehicle. Regarding the nature of the LA molecule, the investigation of physical stability of the emulsions was performed primarily. The following samples were formulated, investigated and compared to placebo M82:M82L2N – the same as placebo with the addition of 10% (w/w) LA and M82L2 – the same as M82L2N with adjusted pH value.

Continual rheology measurements were conducted upon 7 and 60 days of storage and the following parameters were compared: maximum and minimum apparent viscosities (η_{max}, η_{min}), and the hysteresis area (H), generally taken as a measure of system thixotropy (Fig. 5). These parameters were increased in both samples after 60 days which could be attributed to postponed structuring of the systems. Both samples exhibited "shear thinning" pseudoplastic flow behaviour, with pronounced thixotropy; sample M82L2N showed somewhat higher resistance to the applied stress. Initial conductivity values (Table 3)

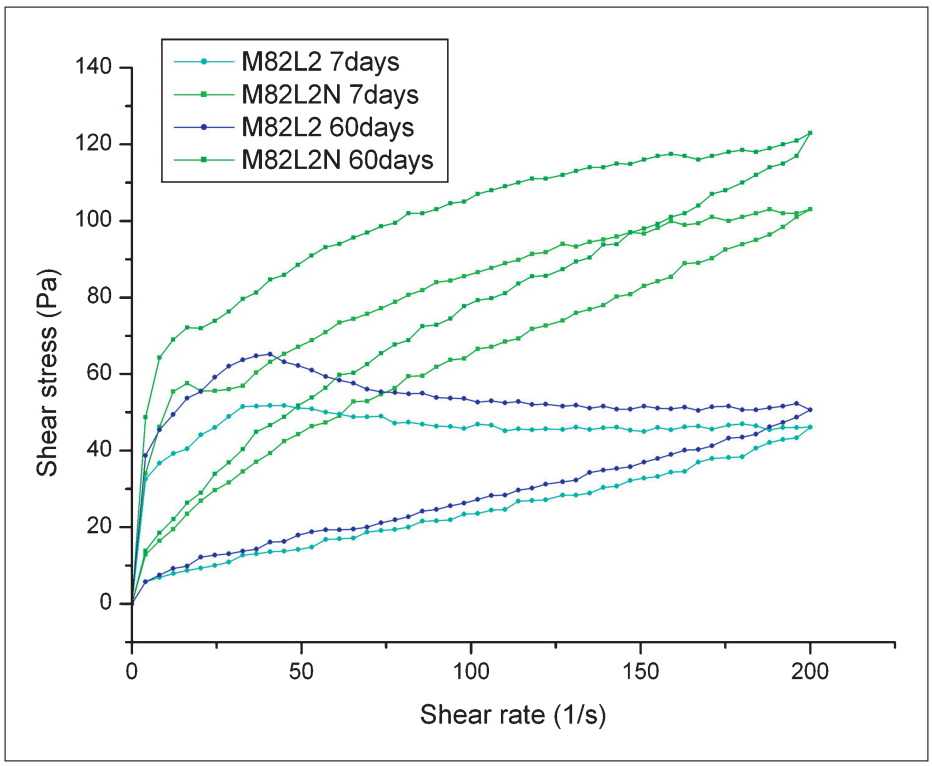

Fig. 5: Viscosity curves of the creams M82L2 and M82L2N 7 vs. 60 days of storage

were relatively high, due to LA presence (as in the first part of the study) with slight increase after 60 days of storage which was greater for M82L2 than for M82L2N, probably due to neutralization and the increased amount of free water in the system. The latter could indicate a slight disturbance of the previously formed lamellar structure seen in sample M82L1 (Fig. 1b) from the first part of the study. Anyway, there were no large differences between the micrographs of the sample M82L1 (6% of LA) from the first part of the study and M82L2 (after 7 days of storage, Fig. 1c) with 10% LA. M82L2 was also additionally stabilized with similar structures as M82L1, which were previously concluded to be lamellar liquid crystals.

In the light of preliminary evaluated physical stability, specified as satisfying for both M82L2 and M82L2N, the APG-based vehicle could be declared as appropriate for 10% LA incorporation, as well as for lower percents of this active which was also shown (Tasic-Kostov et al. 2011). Thus, maintaining the satisfying physical stability may confirm the APG-emulsifiers capability to stabilize the emulsion systems at very low pH values (Table 1), implying that they are proper excipients for formulation with extremely acidic actives such as AHAs. For sure, such a preliminary finding has to be additionally proved, as it is not in line with the claim of some authors that the addition of highly acidic actives causes the APGs hydrolysis (Holmberg 2001).

Having in mind the declared mild LA nature on the one side, and the insufficiency of data describing its overall skin performance on the other, the assessment of skin irritation potential was carried out as an obligatory part of a comprehensive study dealing with a relatively new active substance incorporated into the vehicle based on novel generation of the so called sugar emulsifiers. We performed both *in vitro* and *in vivo* investigation; an in–house three dimensional human skin model, comprising of reconstructed epidermis with functional stratum corneum, was used for *in vitro* study. Effects of the test formulations on cell viability were measured by MTT assay (endpoint for cytotoxicity) (OECD Draft Proposal for a New Guideline, 2008) for M82L2 alongside additional testing for M82 and M82L1 which previously showed satisfying *in vivo* skin irritation profile (Tasic-Kostov et al. 2010). The samples were applied to the ASCs at three different concentrations: 0.25%, 2.5% and 25%. The results for the *in vitro* skin irritation test are presented in Fig. 6a alongside the *in vivo* investigation for M82L2 in Fig. 6b. Samples M82 and M82L1 showed acceptable cell viability, while in the case of M82L2 at the highest formulation concentration (25%) an unacceptable decrease of cell viability (less than 50%) was recorded, pointing to a potential *in vivo* skin irritation effect. However, such formulation concentration (25%), i.e. that quantity, is not usual in real application regime of the emulsions used in the skin treatment. Since *in vivo* investigations showed the absence of skin irritation (Fig. 6b), it could be presumed that both M82L1 and M82L2 posses the satisfying skin safety profile and could be used as skin care intended products.

The results of the moisturizing efficacy of the sample M82L2, compared to placebo M82 are presented in Fig. 7. All participants reported strict compliance with the instructions. The application of both samples resulted in a significant improvement of skin moisture (increase of SCH), compared to both UC and baseline in both short-term (Fig. 7a) and long-term studies (Fig. 7b). Although LA containing sample showed somewhat higher moisturizing capacity, a statistically significant difference between the placebo and sample with LA was not observed. Three hours after the application of the sample M82, significantly higher SCH compared to baseline was measured, whereas the increase of the same parameter for the sample M82L2 was statistically insignificant, indicating even better potential of the sample M82 for prolonged skin hydration.

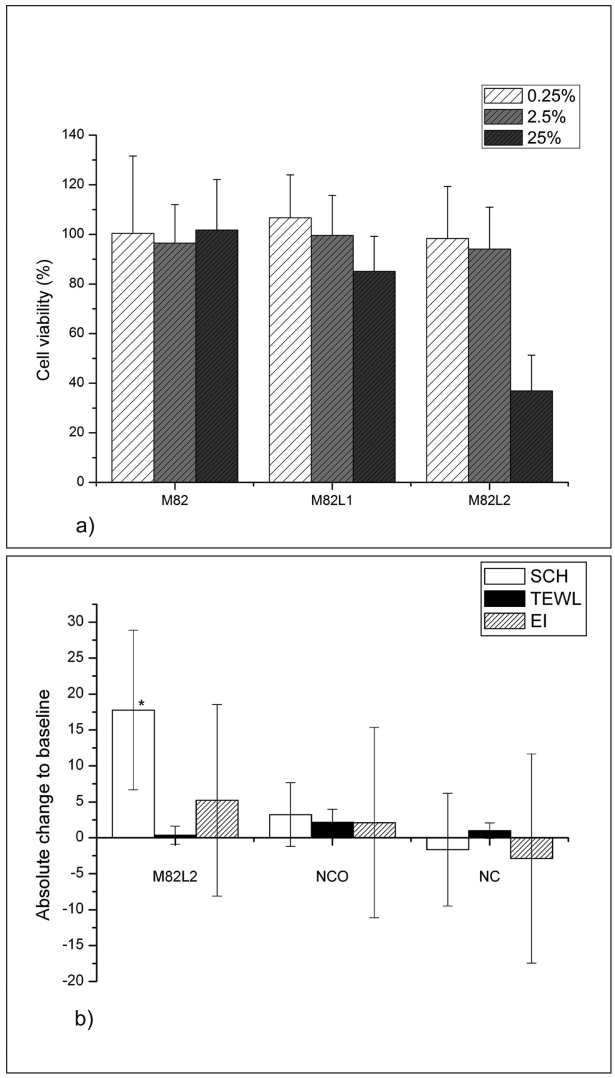

Fig. 6: Skin irritation tests: a) *in vitro*, concentration-viability histograms for the samples M82, M82L1, M82L2; b) the influence of the sample M82L2 on *in vivo* measured skin parameters SCH, TEWL, EI and both controls (under occlusion NCO and without occlusion NC); the results are shown as absolute changes of mean values on the second *vs*. first day and standard error of means

Part II of the study showed that, regarding physical stability and safety assessment, the cetearyl alcohol and coco glucoside based vehicle is a satisfying carrier for higher percents (10%) of LA. The advantageous skin hydrating potential of active vs. placebo is unlikely to be achieved.

Overall, this study showed that C12/14 APG-based vehicles could be promoted as safe cosmetic/dermopharmaceutical vehicles and carriers for extremely acidic and hygroscopic AHA class of actives (specifically LA). Both placebo and LA-containing samples showed prospective safety for human use with the correlation between *in vivo* and *in vitro* findings, confirming that neither APGs nor LA have shown the drawbacks typical of their older-generation representatives, particularly skin irritation and skin barrier impairment potential.

It was shown that LA in concentration of 6% promoted the formation of lamellar liquid crystals, structures which can also hydrate the skin efficiently (Csizmazia et al. 2010), in samples stabilized with cetearyl alcohol and coco glucoside. Still, the extremely hygroscopic nature of LA, which probably withholds the free as well as "depot" water in the sample making it unavailable for skin delivery, affected unexpectedly skin hydration potential of both samples with 6% and 10% of LA.

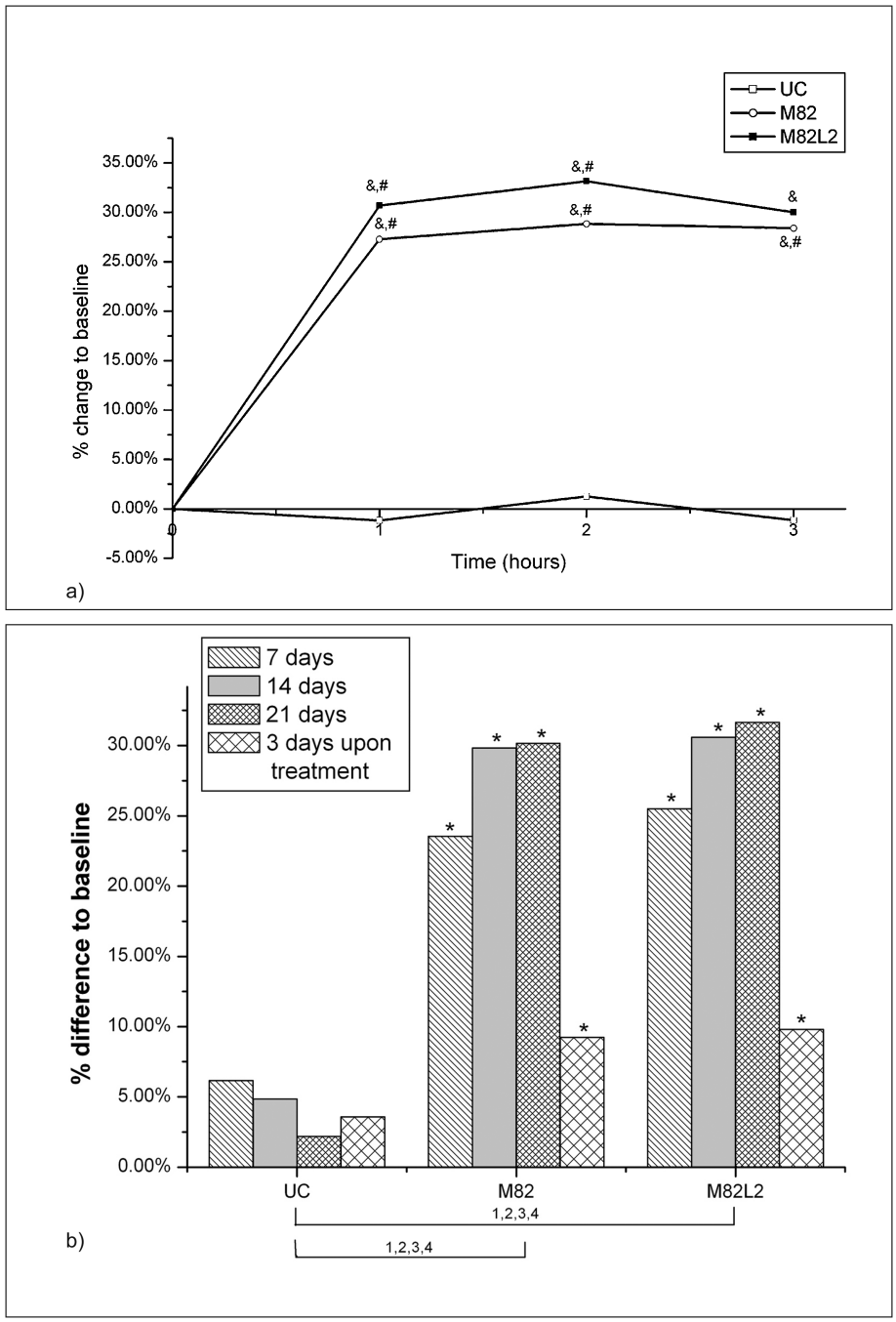

Fig. 7: The effect of topical application of the samples M82 and M82L2 on SCH related to baseline (percent change) in a) short-term study; b) long-term study. Significant differences ($P < 0.05$) being marked with a) (&) for difference to untreated control (UC) and with (#) for difference to baseline in a short-term study; b) (*) for difference to baseline and numbers to mark mutual differences (1) after 7days, (2) after 14 days, (3) after 21 day, (4) upon treatment in a long-term study

This study also underlines the importance of proper choice of ingredients when formulating moisturizing emulsions, since various interactions could significantly impact the skin hydration capacity of even the most effective moisturizing actives. Definitely, it could be summarized that emulsions stabilized by lamellar liquid crystalline structures probably are not satisfying carriers for highly hygroscopic actives in order to reach the full moisturizing potential. However, regarding our results on the colloidal structure of the active samples, it can be presumed that if sufficiently high concentration gradient of water occurs after application (e.g. when applied to extremely dry skin), both LA and APG-based emulsions could expose their full moisturizing potential; prolonged hydration of the skin could occur thanks to the presence of lamellar liquid crystalline structures and potential release of free and bound water.

Safe and effective use on extremely dry skin which requires moisturizing therapy is presumed and should be investigated additionally.

3. Experimental

3.1. Materials

A multiphase oil/water (o/w) "placebo" cream based on Montanov® 82 sugar emulsifier in the concentration of 7% (w/w) was formulated alongside active creams M82L1N, M82L1, M82L2N and M82L2. The samples contained 20% (w/w) of multicomponent oil phase (isopropylmiristate, caprylic-capric triglycerides, decyl oleate, mineral oil, cetearyl alcohol and dimethicone). When corrected, the pH value was adjusted to 3.5–4.0 with the addition of 99% pure triethanolamine (TEA). All samples were adequately preserved using Euxyl K® 300 (Schülke & Mayr, Germany) preservative blend. The humectant used was glycerol at 2% (w/w). For co-stabilisation, a novel co-

emulsifier Montanov®14 (INCI/myristyl alcohol and myristyl glucoside) was used in the concentration of 1.5% (w/w) in all samples. The emulsifier was kindly provided by Seppic, France. Lactobionic acid was kindly provided by Global Calcium, India.

For the cell culture experiments, human dermal fibroblasts, obtained from Cascade Biologics (Mansfield, UK), from the foreskin of newborns were used, cultured according to standard conditions and used from the third to the twelfth passage. Immortalised keratinocytes from the HaCaT-cell line (Human adult, low Calcium, elevated Temperature) were used according to a standard cell culture method during passages 68–84 (Freshney 1994). For cytotoxicity (MTT) experiments, 3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma, Steinheim, Germany), sodium dodecylsulphate (Acros, B-Geel), isopropanol (Riedel de Haën, Seelze, Germany), hydrochloric acid (Merck, Darmstadt, Germany) and freshly double distilled water were used.

3.2. Sample preparation

Placebo sample was prepared by heating emulsifiers and oil at 70 °C and then adding them to the preserved water phase at the same temperature by stirring (stirrer RW16 basic, IKA®WERKE, Germany) at constant temperature for 5 min, and then cooling while mixing. Active samples were prepared by dissolving the LA in the hydrophilic phase of the system just before the mixing of the phases. For neutralized samples, the neutralization was performed 24 h after manufacturing. Test samples were stored for a week prior to the investigation. For the cytotoxicity study, freshly prepared samples, stored for 48 h at 4 °C were employed.

3.3. Colloidal structure characterization

In the first part of the study identification and characterization of the liquid crystalline structures alongside the texture analysis of the samples M82, M82L1 and M82L1N were performed using the following techniques:

3.3.1. pH and conductivity measurements

pH values of the samples were measured by immersing the probe directly into the sample (pH meter HI 8417, Hanna Instruments, Woonsocket, RI). In order to evaluate the water distribution mode within the emulsion system, conductivity values were measured using the conductivity meter (HANNA Hi 98311, Hanna Instruments, Woonsocket, RI).

3.3.2. Polarized light microscopy (PLM)

The mesomorphic structure of the samples was assessed with Zeiss, type III, photomicroscope (Oberkochen, Germany) using cross-polarisers and a wavelength (λ) plate. Relevant images were digitalized using a camera attached to the microscope (Olympus DP12, Japan) and computer software (Olympus DP Soft, version 3.2).

3.3.3. Freeze-fracture transmission electron microscopy (TEM)

To perform a deeper insight into the colloidal structure, TEM micrographs (Leo 922, LeoD-Oberkochen, Germany) of sample replicas (made by the freeze fracture technique) were taken. The samples were shock-frozen in melting nitrogen at 63K between two flat gold holders, fractured at 173K in a BAF 400 instrument (Balzers, Germany) and then shadowed with platinum/carbon (2 nm) at 45° and with pure carbon at 90° for replica preparation. After cleaning with a chloroform–methanol mixture (1:1), the replicas on uncoated grids were fixed onto a sample holder, placed in the vacuum chamber of transmission electron microscope and viewed under a low vacuum at 200 kV.

3.3.4. Rheological measurements

Continual and oscillatory measurements were performed in triplicate, using rotational rheometer (Rheolab MC 120, Paar Physica, Germany); for all samples, the cone and plate measuring system (diameter 50 mm, 1 angle, gap 50 μm, 10 s of sample rest) with a sample thickness of 0.030 mm was used at 20 ± 0.2 °C. During continual testing, a controlled shear rate procedure was applied (shear rate 0.29–200 1/s and back to the start point, each stage lasting 120 s). Oscillatory measurements were conducted in order to determine the linear viscoelastic region of the sample (amplitude sweep), at a constant frequency of 1 Hz and an amplitude sweep ramp of 0.5–30 Pa. A frequency sweep ramp from 0.1 to 10 Hz was performed at a constant shear stress (6 Pa), which was within the previously determined linear viscoelastic region for all samples. The values of the apparent viscosities (η_{min} and η_{max}), the storage (G') and loss (G'') moduli were employed for the rheological characterization of the samples.

3.3.5. Thermogravimetric analysis (TG)

Aiming to differentiate between the bulk and potentially fixed water, TG was conducted, using a TG 220 with a disk station 5200 H (Seiko, Japan). The measurements (in triplicate) were performed with open aluminium pans in the temperature range of 20–100 °C with a heating rate of 2 °C/min.

3.3.6. Differential scanning calorimetry (DSC)

In order to give a deeper insight into the colloidal structure of the samples and consider the possible interactions between LA and the APG emusifier within the system, DSC measurements were carried out. This investigation of the phase transition behavior was conducted using a Differential Scanning Calorimeter DSC 220 (Seiko, Germany), with closed aluminium pan, employing the heating/cooling program with temperature change rate of 2 °C/min between 20 and 105 °C. An empty aluminium pan served as the reference.

3.3.7. Texture analysis (penetration studies)

For the penetration tests a TA-XT2 Texture Analyser (Stable Micro Systems, UK) was employed. The pre-test speed of a probe was set up at 5 mm/s, the test speed (penetration and withdrawal) at 1 mm/s and the probe depth at 5 mm. The probe used was a plastic cylinder with a diameter of 13 mm.

3.4. Physical stability testing

In the second part of the study the physical stability of the samples M82, M82L2N and M82L2 was evaluated employing the following methods: polarized light microscopy (PLM), pH and conductivity measurements and continual rheology using the same methods as described for the first part of the study. The above measurements were performed after 60 days of storage at room conditions and the results 7 vs. 60 days were compared.

3.5. Manufacture of artificial skin constructs (ASCs) and in vitro acute skin irritation test—a cytotoxicity assay

For the purpose of the second part of the study, artificial skin construct (ASC) was manufactured and a modified version of Mosmanís method (Mosmann 1983) was used for the *in vitro* acute skin irritation test-a cytotoxicity assay as previously described (Savic et al. 2009) for three different concentrations (0,25%, 2,5%, 25%) of the test samples.

3.6. In vivo skin performance

To assess possible skin irritation effects of the samples M82 and M82L2, an *in vivo* bioengineering study was performed. Skin parameters TEWL, EI, SCH were measured prior to (baseline values) and 60 min upon cessation of 24-h occlusive treatment. 10 healthy female volunteers (25.4 ± 1.6 years) were recruited. The flexor side of the left forearm was treated with placebo while the right forearm was treated with the M82L2 using precisely delineated and marked cardboard ruler (with empty spaces in the form of rectangles, 16 cm^2 each). Two additional sites were left as a non-treated control under occlusion (NCO) at the right forearm and without occlusion (NC) at the left. Samples were applied in quantities of 0.016 g/cm^2, spread vigorously with rubber glove, and immediately covered with Parafilm® and than with cotton adhesive tapes. All parameters were measured according to the published guidelines and documents (Berardesca 1997; Pinagoda et al. 1990).

To estimate the moisturizing efficacy of same samples by measuring SCH, an additional group of 16 healthy volunteers (24.2 ± 1.6 years) was recruited. They all took part in short- (3 h) and long-term (24 days) trials. The flexor side of their left forearm was treated with the sample M82 and the right one with the M82L2 using cardboard ruler as described. One rectangle was left as an untreated control (UC) on the right forearm.

The amount of applied samples in short-term trial on skin was 0.016 g/cm^2, and measurements conducted were: baseline, 1, 2, and 3 h after application. Then, precise guidelines were given to volunteers to continue the application of the samples. Long-term study measurements were: baseline, after 7, 14, 21 days as well as 3 days upon treatment. *In vivo* measurements were performed in accordance with the Declaration of Helsinki, and the volunteers signed a written consent. They were informed of the study and instructed not to use any skin cleansing or skin care product on the test sites for the whole week before the study, and during the experiment. The study was approved by the local Ethical Committee on Human Research. All subjects had healthy skin and no known allergy to any ingredient of the samples. Before any measurements were taken, the subjects were asked to acclimatize for 30 min under controlled conditions (21 ± 1 °C and 50 ± 5% RH). TEWL was measured using Tewameter®TM 210, EI using Mexameter®MX 18, and SCH by means of Corneometer®CM 825 (all Courage + Khazaka, Germany).

3.7. Statistical analysis

All data were presented as means +/− standard error of the means (SEM). *In vivo* measured parameters (EC, TEWL, EI) were expressed as absolute changes to baseline (Δ values) second *versus* first day for irritation testing and the measured values for M82 and for active sample M82L2 were compared mutually and related to NCO and NC, using one-way ANOVA, followed by Tukey's *t*-test, where appropriate.
For moisturizing efficacy testing SCH was presented as percent change compared to baseline for M82, M82L2 and UC. The data from different sites were analyzed using one-way ANOVA, followed by Tukey's test where appropriate in both short-and long-term efficacy studies. SCH for the same sample in distinct time points was compared using paired sample t-test.
The differences were accepted as statistically significant at $p < 0.05$. Statistical analysis was performed with commercial statistical software SPSS for Windows 13.0.

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References

Al-Bawab A, Friberg SE (2004) Amphiphilic association structures in a model skin lotion with hydroxy acids. Int J Cosmet Sci 26: 139–147.

Bárány E (2000) Human *in vivo* skin irritancy testing. In: Loden M, Maibach HI (eds.) Dry Skin and Moisturizers, 1st ed., Boca Raton: CRC Press, p. 175–182.

Bárány E, Lindberg M, Loden M (2000) Unexpected skin barrier influence from nonionic emulsifiers. Int J Pharm 195: 189–195.

Berardesca E (1997) EEMCO guidance for the assessment of stratum corneum hydration: electrical methods. Skin Res Techno 3: 126–132.

Briden ME, Green B (2005) The next generation hydroxy acids. In: Draelos ZD (ed.) Cosmeceuticals, 1st ed., New York, Elsevier Saunders, p. 205–211.

Csizmazia E, Budai-Szűcs M, Erős I, Makai Z, Szabó-Révész P, Varju G, Csányi E (2010) Thermoanalytical method for predicting the hydration effect permanency of dermal semisolid preparations. J Therm Anal Calorim 102: 313–316.

Fairhust CE, Fuller S, Gray J, Holmes MC (1998) Lyotropic Surfactant Liquid Crystals. In: Demus D, Goodby J, Gray GW, Spiess HW, Vill V (eds.) Handbook of Liquid Crystals, Wiley-VCH Verlag GmbH, p. 341–392.

Fluhr JW, Cavallotti C, Berardesca E (2008) Emollients, moisturizers, and keratolytic agents in psoriasis. Clin Dermatol 26: 380–386.

Freshney RI (ed.) (1994) Culture of animal cells: A manual of basic technique, 3rd ed., New York, p. 113–120.

Green B, Yu RJ, Van Scott EJ (2009) Clinical and cosmeceutical uses of hydroxyacids. Clin Dermatol 27: 495–501.

Holmberg K (2001) Natural surfactants. Cur Opin Colloid Interf Sci 6: 148–159.

Junginger HE (1997) Multiphase emulsions. In: Riegr MM, Rhein LD (eds.) Surfactants in Cosmetics, Marcel Dekker, New York, p. 155–182.

Korhonen M, Hellen L, Hirvonen J, Yliruusi J (2005) Determination of optimal combination of surfactants in creams using rheology measurements. Int J Pharm 197: 143–151.

Loden M (2005) The clinical benefit of moisturizers. J Eur Acad Dermatol Venereol 19: 672–688.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55–63.

Nesseem D (2001) Formulation and evaluation of itraconazole via liquid crystal for topical delivery system. J Pharm Biomed Anal 26**:** 387–399.

OECD, (2008). OECD Guideline for the Testing of Chemicals, Draft Proposal for a New Guideline: *In vitro* Skin Irritation: Human Skin Model Test. Available at: http://www.oecd.org/searchResult/0,3400,en-2649-201185-1-1-1-1-1,00.html.(accessed 15.11.08).

Pinagoda J, Tupker RA, Agner T, Serup J (1990) Guidelines for transepidermal water loss (TEWL) measurements. Contact Dermatitis 22: 164–178.

Savic S, Tamburic S, Savic M, Cekic N, Milic J, Vuleta G (2004) Vehicle-controlled effect of urea on normal and SLS-irritated skin. Int J Pharm 271: 269–280.

Savic S, Weber C, Savic M, Muller-Goymann CC (2009) Natural surfactant-based topical vehicles for two model drugs: Influence of different lipophilic excipients on *in-vitro*/*in vivo* skin performance. Int J Pharm 381: 220–230.

Stubenrauch C (2001) Sugar surfactants-aggregation, interfacial and adsorption phenomena. Cur Opin Colloid Interf Sci 6: 160–170.

Tasic-Kostov M, Savic S, Lukic M, Tamburic S, Pavlovic M, Vuleta G (2010) Lactobionic acid in a natural alkylpolyglucoside-based vehicle: assessing safety and efficacy aspects in comparison to glycolic acid. J Cosmet Dermatol 9: 3–10.

Tasic-Kostov M, Reichl S, Lukic M, Jaksic I, Savic S (2011) Two alkyl polyglucoside natural surfactants varying in the chain lenght in stabilization of lactobionic acid containing emulsions: physicochemical characterization and *in vitro* irritation potential assessment. Riv Ital Sosstanze Grasse: In Press.

Vinardell MP, Mitjans M (2008) Alternative methods for eye and skin irritation tests: an overview. J Pharm Sci 97: 46–59.

Yu RJ, Van Scott EJ (2004) Alpha-hydroxyacids and carboxylic acids. J Cosmet Dermatol 3: 76–87.