International Journal of Pharmaceutics 381 (2009) 220–230

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

# Natural surfactant-based topical vehicles for two model drugs: Influence of different lipophilic excipients on *in vitro*/*in vivo* skin performance

Snežana Savić[a,*], Christian Weber[b], Miroslav M. Savić[c], Christel Müller-Goymann[b]

[a] *Institute of Pharmaceutical Technology and Cosmetology, Faculty of Pharmacy, Belgrade Vojvode Stepe 450, 11221 Belgrade, Serbia*
[b] *Institut für Pharmazeutische Technologie, Technische Universität Braunschweig, Mendelssohnstr. 1, D-38106, Germany*
[c] *Institute of Pharmacology, Faculty of Pharmacy, Belgrade Vojvode Stepe 450, 11221 Belgrade, Serbia*

ARTICLE INFO

*Article history:*
Received 29 November 2008
Received in revised form 19 May 2009
Accepted 9 July 2009
Available online 16 July 2009

*Keywords:*
Natural surfactant-based topical vehicles
Alkylpolyglucoside
Mixed emulsifier
Medium chain triglycerides
Decyl oleate
Isopropyl myristate
Diclofenac sodium
Caffeine
*In vitro* permeation
Cytotoxicity assay
*In vivo* skin performance

ABSTRACT

This study focuses on the properties of topical vehicles based on alkylpolyglucoside natural surfactant-mixed emulsifier, cetearyl glucoside and cetearyl alcohol, in order to propose their use as "ready to use" pharmaceutical bases for a number of model drugs. We were interested to investigate how the alternative use of three lipophilic excipients (Ph. Eur. 6.0), differing in their polarity indexes (medium chain triglycerides (MG), decyl oleate (DO), and isopropyl myristate (IPM), respectively), affects the colloidal structure of the alkylpolyglucoside-based vehicles and *in vitro* permeation profiles of two model drugs: diclofenac sodium (DC) and caffeine (CF), both sparingly soluble in water. Finally, we aimed to evaluate the safety profile of such vehicles *in vitro* (acute skin irritation test using a cytotoxicity assay), comparing it with *in vivo* data obtained by the methods of skin bioengineering.

The results have shown that the emulsion vehicles consisted of a complex colloidal structure of lamellar liquid crystalline and lamellar gel crystalline type. Varying of lipophilic excipient influenced noteworthy variations in the colloidal structure demonstrated as different rheological profiles accompanied to the certain degree by different water distribution modes, but notably provoked by drug nature (an amphiphilic electrolyte drug vs. nonelectrolyte). *In vitro* permeation data obtained using ASC membranes in an infinite dose-type of experiment stressed the importance of the vehicle/solute interactions in case of small variation in formulation composition, asserting the drug properties in the first hours of permeation and rheological profile of the vehicles in the later phase of experiment as decisive factors. *In vitro* skin irritation test demonstrated a mild nature of the emulsifying wax and the absence of negative effects of used oil phases on cell viability in formulation concentrations correspondent to the therapeutic need. This result alongside with data obtained from *in vivo* study, could additionally promote investigated topical vehicles as prospective "ready to use" pharmaceutical bases.



## 1. Introduction

Emulsion systems used in dermopharmacy as drug carriers have to fulfill a number of requirements, e.g. acceptable physical stability, chemical inertness, satisfactory safety profile and drug delivery efficacy (cf. Refai and Müller-Goymann, 2002), reaching at the same time optimal sensory attributes (cohesiveness, spreadability, i.e. rub-out and rub-in and after-feel sensations. . .) (Smith et al., 2002). Most of them are based on traditional ionic or ethoxylated non-ionic emulsifiers or their mixtures with long chain fatty alcohols (so-called mixed emulsifiers). For example, European Pharmacopoeia 6.0 (Ph. Eur. 6.0) recognizes only two mixed emulsifiers, both of them of anionic type: cetostearyl alcohol (type A), emulsifying and cetostearyl alcohol (type B), emulsifying, the first one containing minimum 7% (w/w) of sodium cetostearyl sulphate (SCS), and the second one minimum 7% (w/w) of sodium lauryl sulphate (SLS). The latter surfactant is well established as cytotoxic marker chemical (OECD Draft Proposal for a New Guideline, 2008) and *in vivo* proved skin irritant (Fluhr et al., 2001). While vehicles based on these mixed emulsifiers meet general requirements for pharmaceutical bases, their use is definitely accompanied by adverse skin reactions (Bárány et al., 2000), or associated with unpleasing appearance and unacceptable skin feeling during application (Al-Bawab and Friberg, 2006). Consequently, overcoming the above problems is an important formulation task, which may be accomplished by adequate selection of an emulsifier system (Bárány et al., 2000; Williams and Barry, 2004).

In other words, to promote new simple topical vehicles based on so-called natural surfactant as prospective "ready to use" bases for a number of model drugs, a comprehensive study of their key properties has to be performed. This includes physicochemical characterization of colloidal structure and physical stability study

* Corresponding author.
*E-mail address:* snexs@pharmacy.bg.ac.rs (S. Savić).

0378-5173/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2009.07.007

of the variety of different formulations, investigation of the impact of those formulations on *in vitro* release/permeation and/or *in vivo* efficacy for model drugs, as well as an evaluation of the safety profiles of the formulations.

Therefore, this study focuses on the properties of vehicles based on alkylpolyglucoside natural surfactant-mixed emulsifier, cetearyl glucoside, combined with cetearyl alcohol, that is, an emulsifying wax, in order to propose their use as "ready to use" bases for a number of model drugs. Our previous studies presented a detailed physicochemical and *in vitro*/*in vivo* characterization of a range of vehicles (*placebo samples*), as well as the active samples containing hydrocortisone or urea as model drugs (Savic et al., 2004, 2007).

In this study, we were interested to investigate further how the alternative use of three lipophilic pharmaceutical excipients (Ph. Eur. 6.0), differing in their polarity indexes (medium chain triglycerides (MG), 21.3 mN/m; decyl oleate (DO), 18.7 mN/m and isopropyl myristate (IPM), 24.2 mN/m, respectively), alongside with two additional model drugs, affects the colloidal structure of the alkylpolyglucoside-based vehicles. Furthermore, we have assessed the *in vitro* permeation profiles of following model drugs: a salt of a weak acid (diclophenac sodium (DC), 1% (w/w)), known as an amphiphilic drug, and a weak base (caffeine (CF), 2% (w/w)), both sparingly soluble in water, aiming also to relate the physicochemical properties (water distribution mode and rheological behaviour) of the vehicles with their *in vitro* permeation through the reconstructed human skin models (artificial skin constructs, ASCs). In addition, an evaluation of the safety profiles of active samples *in vitro* was performed, using an alternative method for acute skin irritation test (a cytotoxicity assay) (Spielmann et al., 2007; Vinardell et al., 2008) and *in vivo* (test vehicles), employing the methods of skin bioengineering (Bárány, 2000b). *In vivo* parameters assessed prior and upon 24 h-treatment under occlusion, were: SC hydration (SCH) and transepidermal water loss (TEWL), as a measure of skin barrier properties and skin erythema index (EI), as an indicator of vehicle's irritant potential.

Overall, the study aim was to assess model topical vehicles formed by natural mixed emulsifier, varying in the lipophilic phase, as prospective pharmaceutical ("ready to use") bases for two representative drugs evaluating their colloidal structure and *in vitro/in vivo* skin performance.

## 2. Materials and methods

### 2.1. Materials

The alkylpolyglucoside mixed emulsifier – cetearyl glucoside and cetearyl alcohol – recently FDA certified as pharmaceutical excipient alkyl glucoside (Sepineo SE® 68, kindly donated by Seppic, France) was used in a fixed concentration of 7% (w/w) for the preparation of bases without active ingredient ("placebo") of three model creams, labeled as follows: MG-PL, a basic formulation with 17% (w/w) of medium chain triglycerides, DO-PL and IPM-PL with the same amounts of decyl oleate and isopropyl myristate, respectively, with addition of preserved double-distilled water up to 100% (w/w). All excipients were of pharmacopoeial quality (Ph. Eur. 6.0). Active samples contained dissolved model drugs: 1% (w/w) of diclofenac sodium (DC) (Merck, Germany) or 2% (w/w) of caffeine (CF) (Merck, Germany), respectively. Depending on the incorporated drug, active samples were assigned as: MG-DC, DO-DC and IPM-DC (with diclophenac sodium) or MG-CF, DO-CF and IPM-CF (with caffeine).

For the cell culture experiments, human dermal fibroblasts from the foreskin of newborns were used. These cells were obtained from Cascade Biologics (Mansfield, UK), 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.

### 2.2. Methods

#### 2.2.1. Preparation of samples

Placebo samples (MG-PL, DO-PL and IPM-PL) were prepared by heating the emulsifier and oil at 70 °C in sealed glass vial, and adding the blend to the water phase, by stirring at constant temperature for 3 min (700 rpm), then 3 min at 500 rpm. Upon emulsification/solubilization, cooling was started whilst mixing at 500 rpm (1 min), then at 300 min to the room temperature. Active samples were manufactured by dissolving of the model drug in the hydrophilic phase of the system, using agitation and heating up to 70 °C. Test samples were stored for a week prior to the physicochemical investigation. For the *in vitro* permeation study, cytotoxicity assay and *in vivo* experiments, freshly prepared unpreserved samples were employed, after being stored for 48 h at 4 °C.

#### 2.2.2. Microscopy

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

To perform a deeper insight into the colloidal structure of tested samples, a number of TEM micrographs (Leo 922, Leo D-Oberkochen, Germany) of sample replicas (made by the freeze-fracture technique) were taken. In a typical experiment, the samples were shock-frozen in melting nitrogen at 63 K between two flat gold holders. The frozen samples were fractured at 173 K in a BAF 400 instrument (Balzers, Wiesbaden, 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.

#### 2.2.3. Wide-angle X-ray diffraction (WAXD)

To obtain structural information on the test samples, short-range ordering was examined using WAXD measurements. Diffraction patterns were collected using an X-ray goniometer PW-1050/25 (Philips), coupled with a Xe-filled linear counter (Fuji, Japan). X-rays were produced by an X-ray generator PW-1730 (Philips) using a copper anode (anode current 25 mA; $\lambda$ 0.154 nm, accelerating voltage 40 kV).

From diffraction angle theta ($\theta$), the intermolecular distances were calculated according to the Bragg's law. For each sample measurements were performed twice.

#### 2.2.4. Rheological measurements

Continuous and oscillatory measurements were performed in triplicate on all active samples, using CSR/CSS Rheometer (Bohlin Instruments, Pforzheim, Germany). The following conditions were used for all experiments: cone and plate measuring system (diameter 40 mm, angle 1°), with a sample thickness of 0.030 mm, at $20 \pm 0.1$ °C. During continual testing, a controlled shear rate procedure was applied (shear rate from 0.29 to 200 1/s and back, each stage lasting 120 s).

Oscillatory measurements were conducted in order to determine the viscoelastic region of the sample (amplitude sweep), at a constant frequency of 1 Hz and an amplitude sweep ramp of 0.5–50 Pa. A frequency sweep ramp from 0.1 to 10 Hz was performed at a constant shear stress (10 Pa), which was within the previously determined linear viscoelastic region for all samples. The continuous flow behaviour, the elastic – storage (G′) and viscous – loss (G″) moduli, as well as the complex viscosity ($\eta^*$) were employed for the rheological characterization of the tested samples.

*2.2.5. pH and conductivity measurements*

pH values were measured by immersing the probe directly into the sample (pH meter HI 8417, Hanna Instruments, Woonsocket, RI). In order to determine the type of emulsion and consequently the mode of water distribution within the system, conductivity values were measured using the conductivity meter CDM 230 (Radiometer, Copenhagen, Denmark). All measurements were done in triplicate.

*2.2.6. Thermogravimetric analysis (TGA)*

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

*2.2.7. Manufacture of artificial skin constructs (ASCs)*

According to a previously described method (Winkler and Müller-Goymann, 2002; Hoffmann and Müller-Goymann, 2005) collagen type I was extracted in acetic conditions from rat tails in order to form the dermal collagen equivalent, in which human fibroblasts were incorporated at the concentration of 12,500 cells/ml. For permeation experiments, the collagen gel was then poured into a 6-well-transwell (Corning, Amsterdam, The Netherlands) at a volume of 4 ml/ASC. After one week, the HaCaT cells were seeded onto the contracted matrix (300,000 cells/ASC) and lifted to the air–liquid interface after another week of incubation. ASCs were ready to use after staying 14 days at the air–liquid interface.

For cytotoxicity measurements, the above procedure was altered by pouring the collagen gel into a 12-well-transwell at a volume of 1 ml/ASC and by seeding HaCaT cells onto the contracted matrix in the quantity of 75,000 cells/ASC.

*2.2.8. In vitro permeation study through ASC*

*In vitro* permeation studies were carried out with the modified Franz cells ($n = 6$). The donor compartment was filled with the formulation (infinite dose), whereas the acceptor phase was a phosphate buffer (PBS) pH 7.4, permanently stirred with a rotating magnet (400 rpm). The acceptor compartments of the Franz cells were mounted in a water bath at 37 °C, while the temperature of ASC membrane was 32 ± 0.1 °C. Aliquots of 250 μl were taken over 30 h and replaced with the same amount of fresh PBS to maintain the sink conditions throughout the experiment.

DC and CF concentrations were determined by high performance liquid chromatography (HPLC) using Waters 515/717plus/486 HPLC system (Waters, D-Eschborn), with data analysis by Waters Millennium 32 Chromatography Manager Software and Microsoft Excel 2007.

For the DC assessment, a mixture of water, acetonitrile and glacial acetic acid (40:60:2 (v/v)) was used as an eluent at a flow rate of 1.6 ml/min on a Hypersil ODS column (5 μm, 125 mm × 4 mm with a guard column, Grom, D-Rottenburg-Hailfingen), which served as a stationary phase. The DC peak eluated after 9 min and was detected at λ = 276 nm. Calibration was performed between 1 and 10 μg/ml ($R^2 = 0.9999$, which was linear according to a weighted ANOVA lack-of-fit-test, and homoscedastic according to Cook–Weisberg-test).

For the CF assessment, a mixture of phosphate buffer pH 2.6 and acetonitrile (90:10 (v/v)) was used as an eluent at a flow of 1.2 ml/min on a Lichrospher 100 RP-18 column (5 μm, 125 mm × 4 mm with guard column, Merck, D-Darmstadt), which served as stationary phase. The caffeine peak eluated after 6 min and was detected at λ = 262 nm. Calibration was performed between 2 and 100 μg/ml ($R^2 > 0.9999$, linear according to a weighted ANOVA lack-of-fit-test, homoscedastic according to Cook–Weisberg-test).

Cumulative amounts of drug penetrating per unit area (g/cm$^2$) were plotted against time (s). The *in vitro* permeation rate or steady-state flux ($J$) was generated from the slope of the linear portion of the curve. Taking into account the concentration (Cs) of DC and CF within different vehicles, permeation coefficients ($P$) for both drugs were calculated as well.

*2.2.9. Determination of partition coefficients*

Partition coefficients were measured in the following way: according to each formulation, a water phase including a dissolved drug was prepared. The content of each model drug (DC or CF) in this phase was measured by HPLC at the beginning and the end of the experiment. For each formulation, the water and corresponding oil phase were put together in a flask and mixed with a magnetic stirrer at the room temperature for 24 h (four flasks for each vehicle). After that, each mixture was centrifuged (8000 rpm, Beckman, Germany) and a drug concentration in the water phase determined. The results obtained were compared with the starting concentrations of drugs. The partition coefficient was expressed as the ratio of the DC or CF concentration in the oil phase and its concentration in the water phase.

*2.2.10. In vitro acute skin irritation test—a cytotoxicity assay*

A modified version of Mosmannïs MTT method (Mosmann, 1983) was used: ASCs were placed into a 12-well plate containing different concentrations of test formulations dispersed in phosphate-buffered saline (PBS). ASCs placed into pure PBS served as a control. After 2 h of incubation, ASCs were rinsed with PBS and transferred into a 24-well plate containing 900 μl MSBM and 100 μl aqueous MTT-solution (0.5%) in each well, and incubated at 37 °C and 5% $CO_2$ for two hours. The supernatant was then removed and the dyed ASCs were discoloured in 500 μl lysis solution containing SDS (2.73 g), hydrochloric acid (32.5%; 3.64 g), water (88.18 g) and isopropanol (905.45 g). 150 μl of the coloured solution were then transferred into a 96-well plate and its absorbance was read at 570 nm using a multiplied reader.

*2.2.11. In vivo skin performance—EI, SCH and TEWL measurements*

To assess possible skin irritation effects of test vehicles containing three different lipophilic excipients an *in vivo* bioengineering study was performed. *In vivo* skin effects were assessed via three skin parameters: erythema index (EI) using Mexameter® MX 18, trans-epidermal water loss (TEWL) using Tewameter® TM 210 and stratum corneum hydration (SCH) by means of Corneometer® CM 825 (all from Courage + Khazaka, Germany). Parameters were measured prior to (baseline values on the first day of the experiment) and 60 min upon cessation of 24-h occlusive treatment (second day of the experiment).

In accordance with the Declaration of Helsinki, 10 healthy female volunteers (22.3 ± 0.6 years) were recruited, informed of the study and asked to sign a written consent. The study was approved by the local Ethical Committee on Human Research. All subjects had a healthy skin and no known allergy to any ingredient of the samples; pregnancy and lactation were exclusion criteria. Participants

were instructed not to use any skin cleansing or skin care products at the test sites for the whole week before the study, as well as during the experiment. Before any measurements were taken, subjects were asked to acclimatize for 30 min under controlled conditions ($21 \pm 1\,^{\circ}C$ and $50 \pm 5\%$ RH).

The flexor side of the left forearm was treated with placebo samples (DO-PL and IPM-PL), while the right forearm was treated with the basic formulation MG-PL, using precisely delineated and marked cardboard ruler (with empty spaces in the form of rectangles, 16 $cm^2$ each). Two additional sites at the right forearm were left as a non-treated control under occlusion (NCO) or without occlusion (NCWO). Samples were applied in quantities of 0.016 $g/cm^2$, and spread over the skin vigorously, using a gloved finger. Immediately after application, the treated sites were covered with Parafilm® and, loosely, with cotton adhesive wraps. All parameters were measured according to the published guidelines and documents (Berardesca, 1997; Clarys et al., 2000; Rogiers, 2001).

#### 2.2.12. Statistical analysis

Whenever applicable, data were presented as mean ± S.D. Parameters from *in vivo* experiments (EI, TEWL, SCH) were expressed as percentage change of the second vs. first day, and plotted as vertical bars with medians, 25th and 75th percentile (10th and 90th percentile as error bars).

*In vitro* permeation and acute skin irritation data were compared by Student *t*-test for independent samples. *In vivo* effects of vehicles with different excipients were compared mutually and related to non-treated controls, under and without occlusion (NCO and NCWO), using Wilcoxon matched paired signed rank test. Statistical significance was set at $p < 0.05$.

## 3. Results and discussion

### 3.1. Colloidal structure of the drug containing samples

In order to fully appraise the new emulsifying wax as promising pharmaceutical excipient, it is necessary to prove its ability to stabilize different emulsion systems, prospective "ready to use vehicles", in fashion similar to that achieved by traditional mixed emulsifiers (Eccleston, 1997; Eccleston et al., 2000). Additionally, it is desirable to exclude some drawbacks typical for traditional mixed emulsifies, like postponed structuring in ethoxylated non-ionic systems (Eccleston, 1997) or irritant potential attributed to anionic surfactants (Bárány et al., 2000). To obtain this information a comprehensive physicochemical study was performed accompanied by evaluation of *in vitro*/*in vivo* skin performance.

*First of all*, polarization microscopy has revealed a lyotropic interaction of lamellar type, but with different features in different samples. Namely, in samples with medium chain triglycerides (MG-DC and MG-CF) and decyl oleate (DO-DC and DO-CF), irrespective of the incorporated drug, anisotropic structures like distorted Maltese crosses (known also as "onion" rings) have been seen (Fig. 1a–d), alongside with well developed lamellar gel phase, particularly in samples with DO. On the contrary, in the samples with IPM (Fig. 1e and f), remnants of the gel surrounding larger and floccules of the smaller droplets are more pronounced than anisotropy at their edges. Although this screening could indicate synergism between the two types of lamellar phases, well established in semisolids stabilized by traditional mixed emulsifiers (Eccleston et al., 2000), it is clear that variation of only one component, a lipophilic excipient, may affect significantly the appearance of the systems based on the same mixed emulsifier.

To obtain a deeper insight into the structure of these colloidal systems TEM and WAXD alongside with rheological and TGA characterization were employed.

From the TEM micrographs of DC-loaded samples (Fig. 2a–c), typical features of the lamellar liquid crystalline, particularly lamellar gel phase, such as planar layers, terraces and steps (Makai et al., 2003; Mondain-Monval, 2005) have been detected, pointing in that way at the synergism of two lamellar phases mentioned above (Eccleston et al., 2000). However, certain differences corresponding to the findings from polarization microscopy are noticed. Thus, in the sample with IPM (IPM-DC) wide strips with occasional sharp edges could be seen (Fig. 2c), which implies a solid structuring of the continual emulsion phase (Makai et al., 2003; Mondain-Monval, 2005). According to our previous studies (Savić et al., 2006; Savic et al., 2007) such picture indicates the predominant existence of the lamellar gel crystalline phase within the system, or more rigid inner arrangement of the lamellae. Similarly to DC-loaded samples in those with CF, some typical signs of the lamellar structures are clearly seen as well (representative micrograph, Fig. 2d), meaning that variation of drug did not affected noticeably the stabilization mechanism developed by emulsifier/water/oil interaction. However, it seems, particularly in the case of IPM-DC sample, that DC amphiphilic nature contributes to possible drug involving to the formed lamellar mesophases, thus modifying its release/permeation profiles.

WAXD patterns additionally prove previous findings. In all cases depicted a single sharp reflection was registered at 0.41–0.42 nm (Fig. 3a–d), placed in the middle of the diffuse halo at 0.45 nm, which confirms the presence of the α-crystalline gel phase gathered as well with lamellar liquid crystalline phase (Fairhust et al., 1998). Overall, obtained WAXD patterns *alongside with* polarization microscopy and TEM findings definitely direct to the structural synergism of liquid crystalline phase ($L_\alpha$) and lamellar α-crystalline gel phase ($L_\beta$) in samples with both model drugs. However, it is expected from inner structure of lamellae to be probably variable connected to different molecular structure of used oils. The latter affects their different packing inside the lamellar sheets causing different level of mobility (or rigidity) of the alkyl chains within these layers and different capability of hydrogen bonding (water entrapping). This could reflect on the rheological behaviour and water distribution mode within the samples that may impart the both water evaporation from the system and therefore hydrating potential of the vehicle as well as the drug delivery to the skin.

Flow curves (Fig. 4a and b) of both groups of the samples reveal plastic behaviour with moderately pronounced thixotropy irrespective of used oil and incorporated drug. Obtained shear-thinning behaviour is desirable property in semisolids, since they should be "thin" during the application and "thick" otherwise (Pena et al., 1994). Nevertheless, CF loaded samples with MG and DO have shown some higher resistance to the applied shear stress related to corresponding DC samples, while in the case of IPM was an opposite situation, implying to the TEM finding and previously commented DC amphiphilic potential. On the other side, certain deviation from described trend was observed when viscoelastic measurements have been carried out.

Viscoelastic rheological behaviour, typical for the lamellar α-crystalline gel network formed by traditional mixed emulsifiers (Robles-Vasquez et al., 1993; Nemeth et al., 1998), has been detected in all test samples (Fig. 4c and d). However, some differences depending on the type of used oil and incorporated drug have been recorded. When take into account the parameters' values at frequency of 1 Hz (data not shown in table), only in sample DO-DC viscous modulus ($G''$, 180 Pa) was higher than elastic one ($G'$, 81.3 Pa), with the lowest $G'/G''$ ratio of 0.45, while in the rest of the samples the opposite finding was registered. Namely, other samples showed predominance of elastic component with the ratio $G'/G''$ ranked as follows: MG-CF (4.30), IPM-CF (3.80), DO-CF (3.0), IPM-DC (2.11) and MG-DC (1.89). However, it should be emphasized that sample MG-DC was with the highest the both, elastic (3170 Pa) and

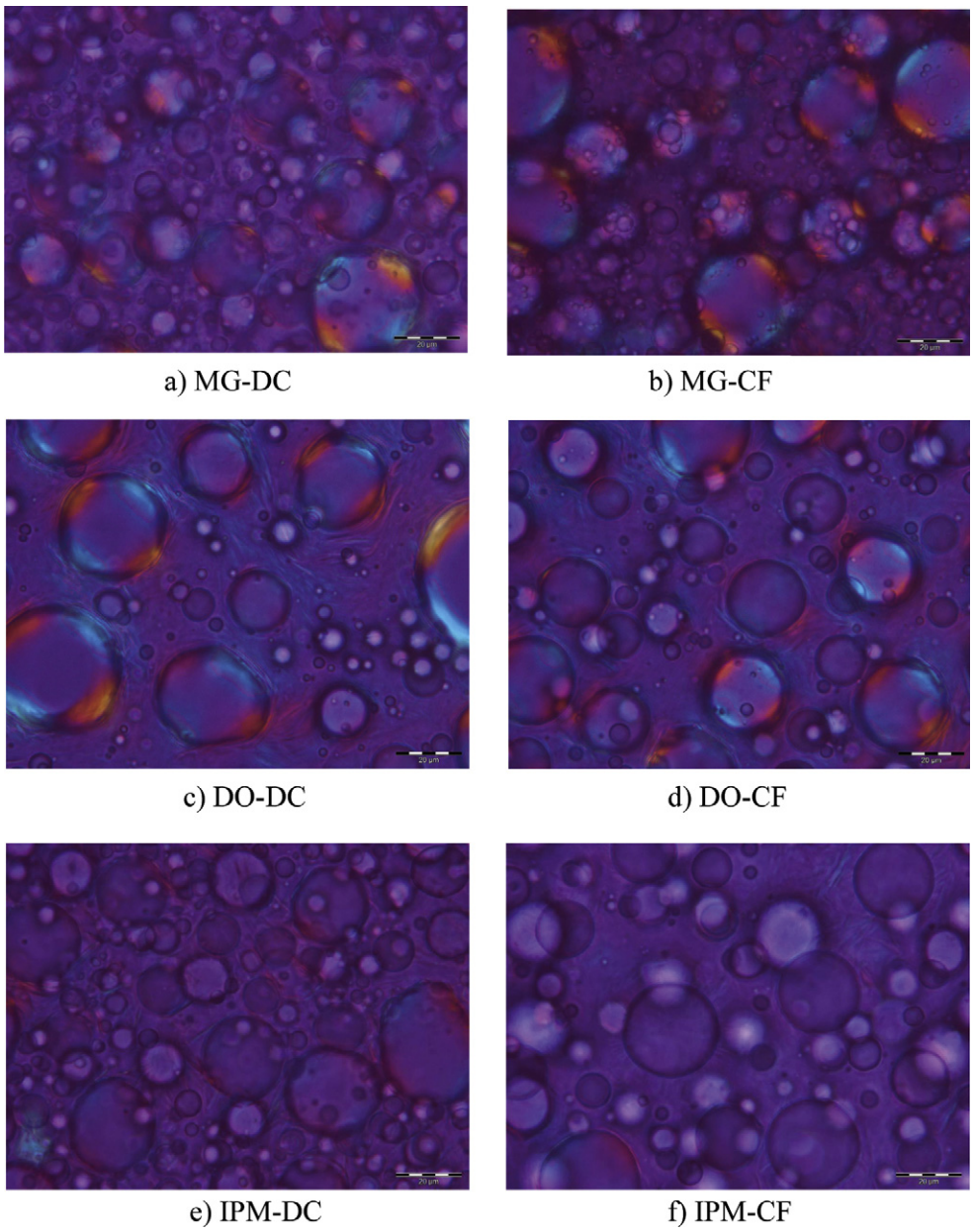


**Fig. 1.** Polarisation micrographs of vehicles based on natural mixed emulsifier (cetearyl glucoside and cetearyl alcohol) varying in lipophilic excipient and model drug: (a) MG-DC; (b) MG-CF; (c) DO-DC; (d) DO-CF; (e) IPM-DC; (f) IPM-CF; bar 20 μm.

viscous (1680 Pa) moduli followed by samples DO-CF (G′, 1500; G″, 500 Pa) and IPM-DC (G′, 1010 Pa; G″ 479 Pa). If we keep in mind the influence of both, the used oil and model drug, there was no a consistent trend in rheological properties of the vehicles. Even so, some implications could be drawn from oscillatory measurements. First of all, if we exclude sample DO-DC, it seems that presence of an amphiphilc ionic type of the drug (mesogen structure of diclofenac sodium), contributes to the rheological performance of semisolids based on cetearyl glucoside and cetearyl alcohol mixed emulsifier (Fig. 4c and d), probably due to drug potential to be inserted within the lamellae of both phases, the gel-crystalline and the liquid crystalline one (Kriwet and Müeller-Goymann, 1993). Furthermore, in these DC samples elastic component was not distinctly higher than viscous one approaching them to the systems predominantly stabilized by α-crystalline gel phase or reflecting, at least, more rigid inner structure of the lamellae, supporting in that way a previous supposition. On the other side, nonelectrolyte drug CF keeps lamellae in more fluid state (more expressed elastic modulus, Fig. 4d), pointing to more pronounced lamellar liquid crystalline phase within CF samples comparing with DC ones. Beside that, in the context of lipophilic excipient influence, in CF samples much lower variations were detected. More precisely, elastic moduli of MG-CF and DO-CF samples have showed almost an overlapping, with close values of viscous moduli as well in whole measuring range (Fig. 4d), while IPM decreased these parameters to the certain extent. Therefore, it could be summarized that in case of nonelectrolyte drug more polar oils may produce more elastic systems based on alkylpolyglucoside-type of mixed emulsifier, less viscous at the same time, indicating to the certain degree a higher presence of lamellar liquid crystalline phase, at least in comparison with corresponding samples containing the ionic amphiphilic drug. Otherwise, all samples irrespective of oil and used drug have exhibited satisfied aesthetic and applicative properties (gloss, texture, spreadability and rub-in sensation. . .), as well as appropriate pH values recommended for products intended for the skin administration (Table 1). It is of interest to emphasize that measured pH

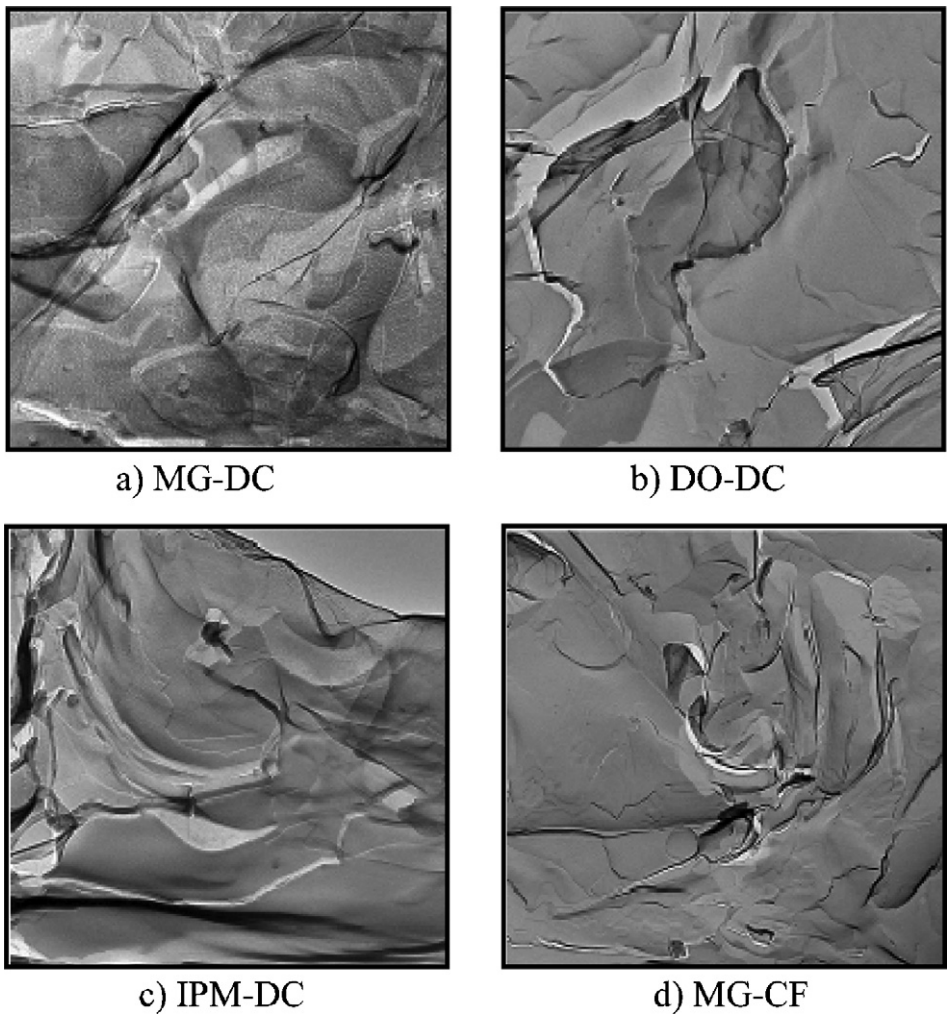


**Fig. 2.** TEM micrographs of vehicles based on natural mixed emulsifier (cetearyl glucoside and cetearyl alcohol) with a different lipophilic excipient and a different model drug: (a) MG-DC; (b) DO-DC; (c) IPM-CF; (d) MG-CF.

values were in the range 4.29–8.08 (Table 1), dependent on used oil phase and incorporated drug. In fact, all samples kept satisfied physical stability, confirming the emulsifier capability of stabilizing the emulsion systems at rather different pH values.

Additional data on the microstructure of the systems were obtained using conductivity measurements and thermogravimetric analysis. In general, test samples with DC had higher conductivities (Table 1), which is due to the ionic nature of the drug. However, there are differences among DC-containing samples (MG-DC vs. DO-DC vs. IPM-DC), which could be explained by different microstructure and ratio of bound (interlamellarly fixed)/free water and related to the rheological data described above. In principle, the conductivity of the multiphase emulsion systems corresponds to the fraction of free water and concentration of the ions in it (Korhonen et al., 2002). Analyzing the samples with DC (Table 1), it appears that sample DO-DC contained the highest amount of bound water, *possibly* together with "entrapped" model drug. This explanation has the sense if correlates conductivity value with TGA results (Table 2). It is obvious that this sample has lost the least amount of water in the first temperature range, which corresponds well with evaporated amount of free or "bulk" water from the system. The huge quantity of the water (the "secondary" one) evaporated from the vehicle in the second temperature range (Table 2), likely due to the melting of the main constituent of lipophilic gel phase (cetearyl alcohol semi-hydrates) (Savic et al., 2007). In two additional DC-containing samples higher conductivities were recorded, indicating either more free water within the samples or more DC ions in it. The latter seems more possible and correlates well with obtained rheological profiles (Fig. 4a and c) and dynamic of water loss from these systems (Table 2) supporting

**Table 1**
pH and conductivity values of emulsion vehicles varied in lipophilic excipient.

| Sample | pH | Conductivity (μS/cm) |
|---|---|---|
| MG-DC | 8.08 | 52.20 |
| DO-DC | 7.76 | 29.58 |
| IPM-DC | 6.32 | 43.75 |
| MG-CF | 5.30 | 8.19 |
| DO-CF | 4.29 | 9.54 |
| IPM-CF | 4.98 | 6.24 |

**Table 2**
Percentage of total (TL) and partial weight losses (PL) over the specified temperature ranges for the vehicles dependent on the oil type.

| Sample | TL (%) | 20–50 °C (%) | 50–70 °C (%) | 70–100 °C (%) |
|---|---|---|---|---|
| MG-DC | 74.97 ± 2.1 | 11.99 ± 0.46 | 57.96 ± 1.37 | 5.02 ± 0.78 |
| DO-DC | 75.27 ± 2.34 | 8.09 ± 0.78 | 64.49 ± 1.23 | 2.69 ± 0.41 |
| IPM-DC | 73.51 ± 1.98 | 12.39 ± 0.56 | 55.33 ± 1.11 | 5.85 ± 0.17 |
| MG-CF | 73.91 ± 2.7 | 17.01 ± 0.31 | 55.21 ± 1.03 | 1.69 ± 0.19 |
| DO-CF | 73.06 ± 2.11 | 19.31 ± 0.49 | 51.80 ± 1.09 | 1.95 ± 0.12 |
| IPM-CF | 75.01 ± 2.43 | 19.40 ± 0.98 | 53.62 ± 1.08 | 1.99 ± 0.09 |

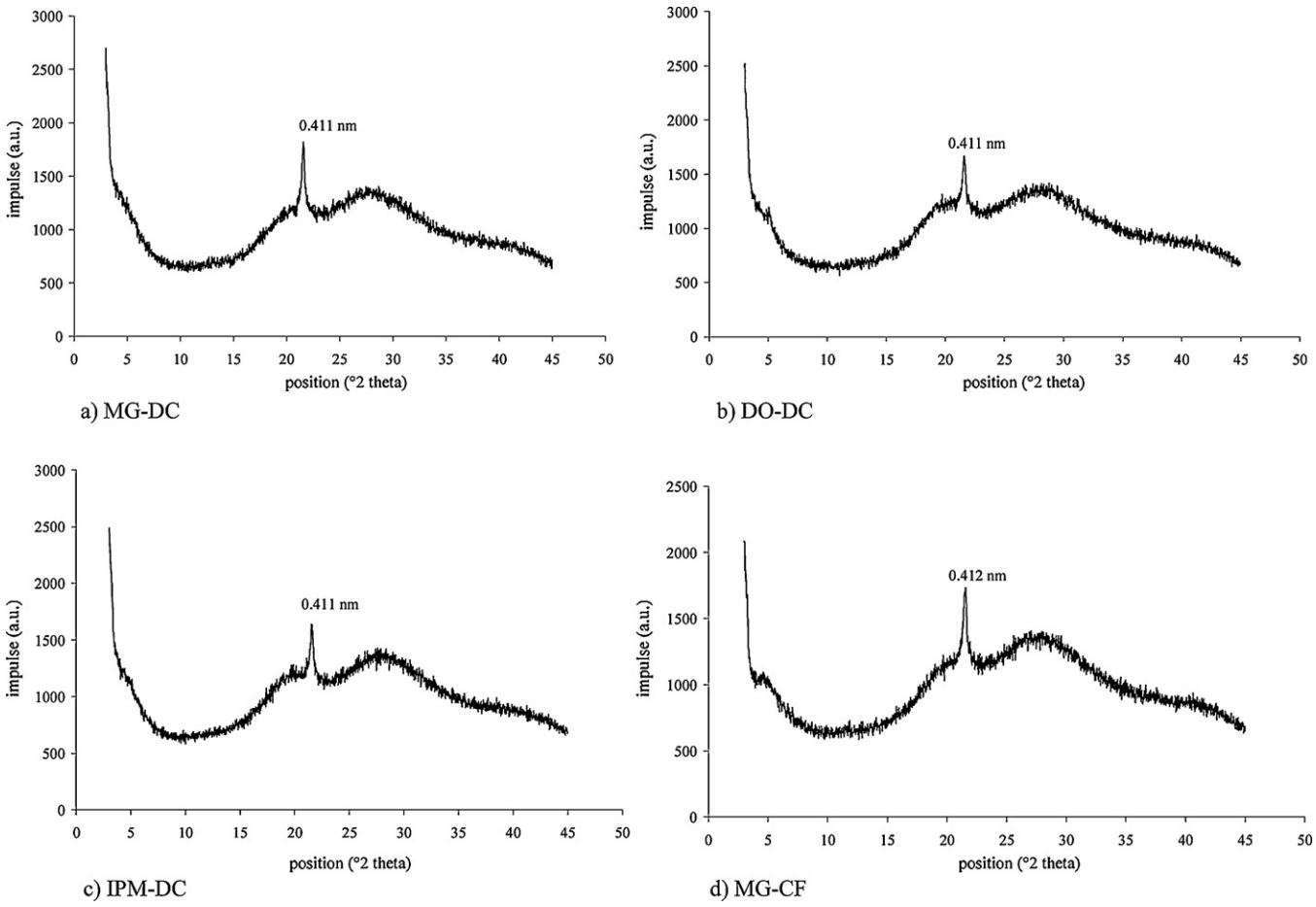


**Fig. 3.** WAXD patterns of vehicles based on cetearyl glucoside and cetearyl alcohol with different lipophilic excipient containing different model drug: (a) MG-DC; (b) DO-DC; (c) IPM-DC; (d) MG-CF.

together the above assumptions on well developed α-crystalline gel phase. In this term, in the samples MG-DC and IPM-DC similar percentages of interlamellarly fixed water have been found (Table 2), pointing again at strong hydrophilic gel. As expected, CF containing samples were with significantly lower conductivities (Table 1), predominantly due to non-ionic drug nature, but also typical for the multiphase emulsion systems (Korhonen et al., 2002). Small variations of conductivities with change of oil phase in the system were in line with TGA and rheological results for corresponding samples.

In summary, there are distinct variations in the colloidal structure of the vehicles based on natural mixed emulsifier containing three different lipophilic excipients, in a great extent influenced by the presence of two different model drugs, an amphiphilic ionic (DC) and the second one of nonelectrolyte type. The differences were demonstrated as different rheological profiles accompanied to the certain degree by different modes of water distribution within the structure, particularly in DC-loaded samples, explained by partial DC integration to the lamellae of created mesophases. The above may affect the drug diffusion through the vehicle and therefore its availability to penetrate the skin. In addition, topical vehicles have the potential to influence the skin condition (hydration, barrier properties) in a variety of manners, thus changing its permeability. It was therefore of interest to assess the permeation of two model drugs from these vehicles and their *in vivo* performance on the skin.

### 3.2. In vitro permeation study through ASCs

The results of the permeation of two model drugs, DC and CF, from six test samples are presented in Table 3 and Fig. 5a and b.

Interestingly, samples DO-DC and DO-CF have shown the lowest values of the steady-state flux (Table 3, $2.40 \pm 0.75 \times 10^{-8}$ g/cm$^2$ s and $5.50 \pm 0.95 \times 10^{-8}$ g/cm$^2$ s, respectively) and consequently the permeation coefficients (Table 3). For two reasons at least this finding deserves an adequate explanation.

Among the CF-containing samples, even though they had similar viscoelastic profiles and close values of complex viscosity (Fig. 4d), sample DO-CF has exhibited significantly lower drug permeation starting from the 6th hour of the experiment, comparing to the other CF-loaded samples (Fig. 5b). On the other side, DC-containing samples, have revealed almost identical permeation profiles during the first 8 h of the experiment (Fig. 5a), despite DO-DC sample had distinctly different rheological profile (Fig. 4c) from two other samples. In both groups, samples containing medium chain triglycerides (MG-DC and MG-CF) have shown the highest total amount of permeated drug after 24 h of experiment, reaching statistical significance in comparison with corresponding samples varying in lipophilic excipient (Fig. 5a and b).

**Table 3**
Partition coefficients (log *P*), flux (*J*) and permeation coefficients (*P*) of DC and CD from samples with different lipophilic excipients.

| Sample | Partition coefficient (log *P*) | $J$ ($\times 10^{-8}$ g/cm$^2$ s) | $P$ ($\times 10^{-5}$ cm/s) |
|---|---|---|---|
| MG-DC | −1.82 | 3.36 ± 1.26 | 3.36 ± 1.26 |
| DO-DC | −0.78 | 2.40 ± 0.75 | 2.40 ± 0.75 |
| IPM-DC | −0.68 | 4.48 ± 1.22 | 4.48 ± 1.22 |
| MG-CF | −0.11 | 9.38 ± 2.69 | 4.69 ± 1.35 |
| DO-CF | −0.91 | 5.50 ± 0.95 | 2.75 ± 0.48 |
| IPM-CF | −0.29 | 9.93 ± 1.85 | 4.48 ± 0.93 |

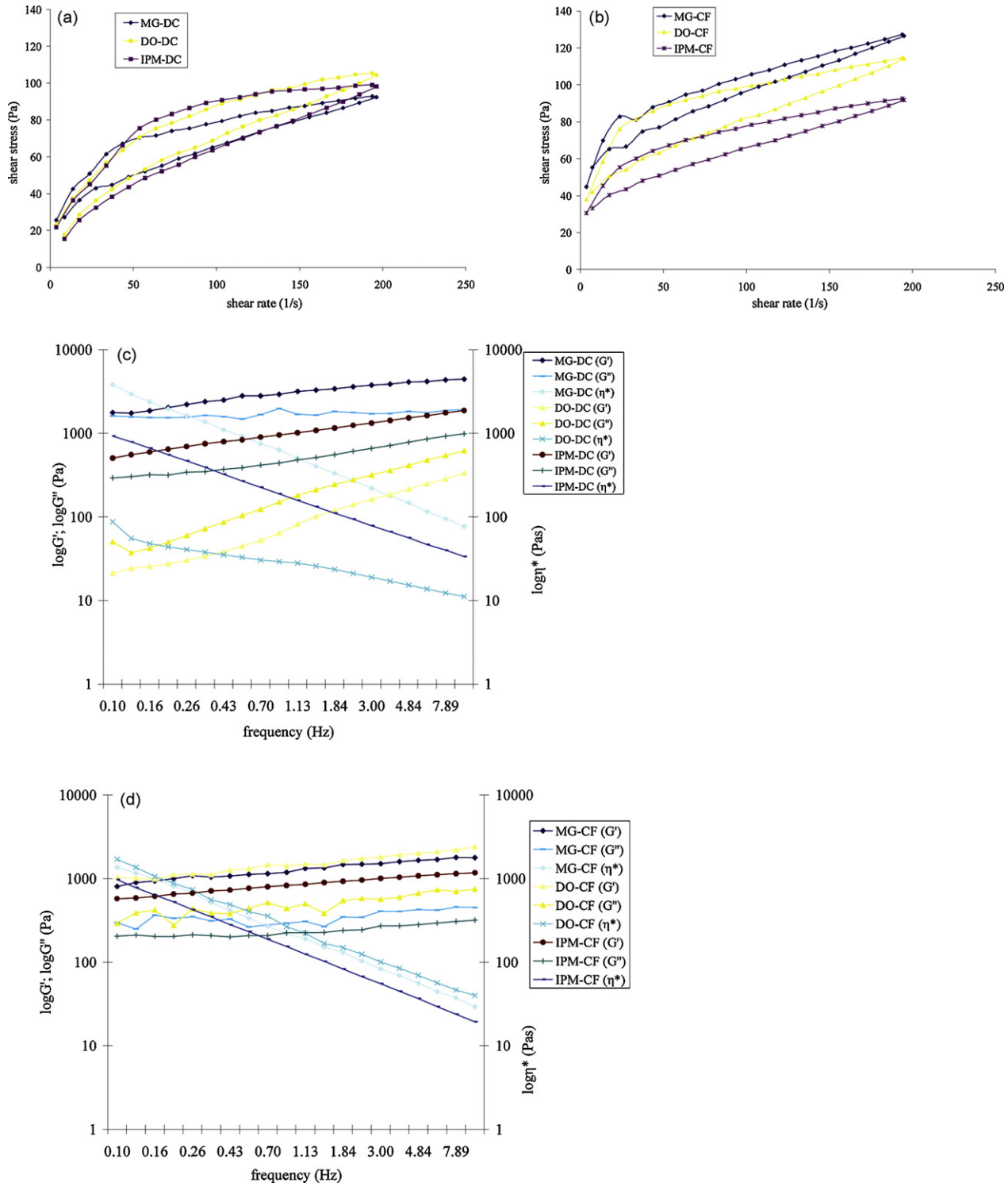


**Fig. 4.** Rheological behaviour of samples containing different lipophilic excipient and model drug: (a) and (b) continual rheological measurements; (c) and (d) viscoelastic measurements.

From the above results, it appears a contradictory situation when considering the relationship between rheological behaviour and drug permeation from samples differing in lipophilic excipients. It is clear that in case of an amphiphilic electrolyte type drug even noticeable differences in rheological parameters of the vehicles did not have a decisive influence on drug permeation, at least in the first hours of experiments. Opposite, samples with nonelectrolyte drug despite having similar rheological properties showed significantly different permeation profiles starting to appear from 6th hour of experiment.

These findings may indicate bimodal nature of vehicle/solute interactions, at least in the infinite-dose approach experiment. On the one hand, this supposition emphasizes the role of drug characteristics in permeation process, if variations in vehicle formulations are not discriminative and vehicle/membrane interactions are excluded. Analyzing drugs' partition coefficients in different vehi-

cles (Table 3), it could be seen that sample MG-DC ($\log P = -1.82$) has the highest percent of drug distributed within the vehicle polar phase among DC-containing samples, while in the second group that is the sample DO-CF ($\log P = -0.91$). On the other side, there were certain differences in colloidal structure of the vehicles, particularly connected to the arrangement of lamellae, which may affect drug diffusion through the vehicle and reflect to its permeation profiles. Here it is of importance to remind that ratio between elastic and viscous modulus was the lowest one in DO-CF sample, in fact this sample was the most viscous (the highest $\eta^*$ as well). All these facts taken together may lead to the conclusion that drug nature including its partition coefficient within the vehicle affects permeation through ASCs in the first hours of experiment, while in later phases of the experiment differences in colloidal structure may be more decisive factor.

It seems reasonable as well that in later phases of experiment permeation profiles may be attributed to the potential interactions between the vehicle components and the skin construct (ASC) membrane, in fact they could not be excluded from the consideration. But, there are disagreements in the literature regarding the validity of the skin constructs in *in vitro* penetration/permeation experiments (Schmook et al., 2001; Ponec, 2002), mostly regarding different barrier characteristics from the human epidermis. Recently published validation study (Schäfer-Korting et al., 2008) proposed employing of three used reconstructed human epidermal models as appropriate alternatives to human skin and pig skin for the assessment of skin permeation and penetration *in vitro*. Study

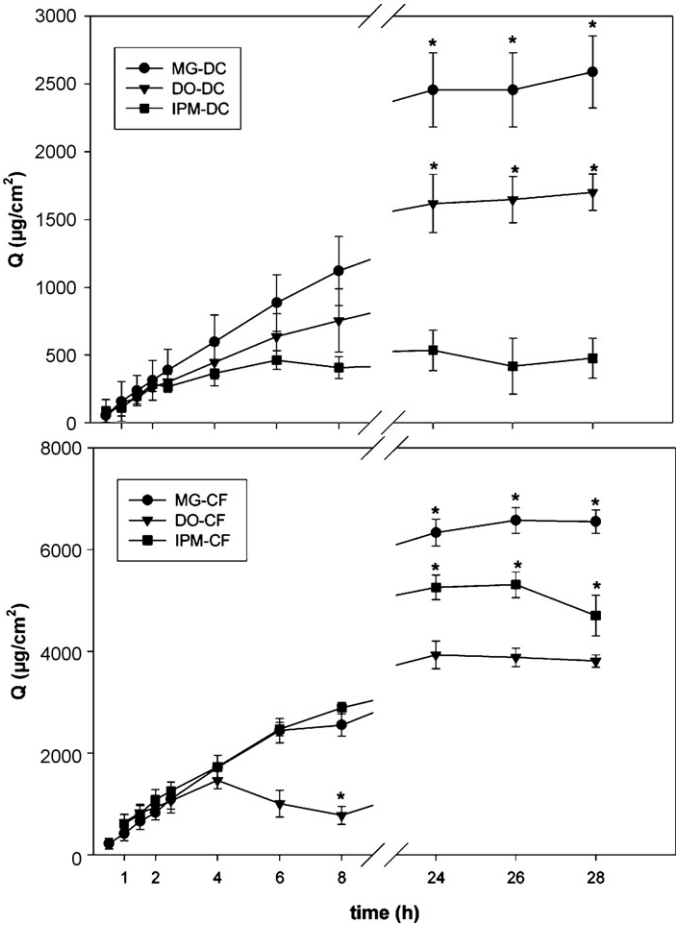


**Fig. 5.** *In vitro* permeation profiles of: (a) 1% (w/w) of DC from different vehicles (MG-DC vs. DO-DC vs. IPM-DC); (b) 2% (w/w) of CF from corresponding vehicles (MG-CF vs. DO-CF vs. IPM-CF) through ASC. *Q*: permeated amount/area (µg/cm²). Bars = ±S.D. $^*p < 0.05$.

was performed with a set of drugs (caffeine was included) in form of aqueous solutions, where obtained results for CF permeation coefficient were highly comparable with ours. However, since simple drug solutions had been used as vehicles there were not considerations on possible vehicle/membrane interactions (Schäfer-Korting et al., 2008). Our results as well may not suggest any interactions of this type, likely due to rather small variation in vehicles' composition.

It can be concluded that permeation profiles of the two model drugs through ASC membrane and from natural surfactant-based vehicles, varying in the lipophilic excipient, are affected by a number of variables, but predominantly those related to the vehicle/solute interactions.

### 3.3. *In vitro acute skin irritation test—a cytotoxicity assay*

Assessment of skin irritation potential is an important part of any comprehensive investigation of a new excipient or novel consumer product intended for skin application (Vinardell and Mitjans, 2008). Furthermore, ECVAM (European Center for Validation of Alternative Methods) in its validation study (Spielmann et al., 2007) endorsed the scientific validity of the EPISKIN™ test as replacement for the rabbit skin irritation method and EpiDerm™ method for identifying skin irritations as well. In this study, an in–house three dimensional human skin model, comprising a reconstructed epidermis with functional stratum corneum, comparable with mentioned above (Schäfer-Korting et al., 2008) was used. Effects of test formulations on cell viability were measured by MTT assay (endpoint for cytotoxicity) (OECD Draft Proposal for a New Guideline, 2008). Samples were applied to the ASCs at three different concentrations: 0.25%, 2.5% and 25%. The aim of this part was twofold: to assess skin irritation potential of the natural surfactant-based mixed emulsifier, generally declared as mild (Mehling and Hensen, 2004), as well as possible irritation effects produced by variation of lipophilic excipients within the vehicles.

The results of the *in vitro* skin irritation test are presented in Fig. 6a and b. All samples show a concentration dependent decrease in ASC cell viability. Even in the case of 25% of the test samples, the cell viability was more than 50% (OECD Draft Proposal for a New Guideline, 2008), ranked in decreasing order as follows: MG-CF $76.42 \pm 9.18\%$ > DO-CF $66.89 \pm 25.29\%$ > DO-DC $64.89 \pm 9.69\%$ > IPM-CF $61.92 \pm 11.14\%$ > MG-DC $56.74 \pm 14.18\%$ > IPM-DC $53.89 \pm 3.75\%$. Although there were not significant differences, this finding may imply the drug dependent drop in cell viability, more pronounced in case of DC-loaded samples. For lower concentrations, 0.25% and 2.5% of test formulations, no significant differences among the rest of the samples were found as well, although the trend of decreased viability in IPM-containing samples was visible.

The cytotoxicity assay results indicate that the samples based on cetearyl glucoside and cetearyl alcohol mixed emulsifier varying in lipophilic excipient are generally safe to use as topical vehicles. Of all used oil phases, IPM, particularly in case of DC-containing sample, has caused almost the borderline cell viability ($53.89 \pm 3.75\%$, OECD Draft Proposal for a New Guideline, 2008), obviously the drug influenced and only just at the highest concentration of test formulation, supporting also the safe use of all three lipophilic excipient in this sort of topical vehicles. However, it is necessary to acquire *in vivo* data in order to draw a definitive conclusion on irritation potential of prospective "ready to use" topical vehicles based on natural mixed emulsifier.

### 3.4. *In vivo skin performance—EI, SCH and TEWL measurements*

The percentage change of all *in vivo* measured parameters (EI, TEWL and SCH), related to the baseline values are given in Fig. 7a–c. A direct assessment of the *in vivo* irritation potential of placebo sam-

ples was carried out via erythema index (EI) measurement after occlusion. In addition, stratum corneum hydration (SCH) and barrier function via transepidermal water loss (TEWL) were measured in order to obtain a complete picture of the skin performance (Fang et al., 2003).

Otherwise, it should be emphasized that evaluation of *in vivo* skin performance was performed for both placebo and all active samples in three groups of healthy human volunteers (each of 10), but with almost identical effects to the measured skin parameters independent of incorporated drug (DC vs. CF). Analyzing these effects, no significant difference was found. Consequently, we concluded that presence and content of used formulation excipients have predominant influence on samples' *in vivo* skin performance and only results for placebo samples are presented.

Throughout the study, no adverse skin reactions related to test samples were recorded. All test samples have shown a decrease in the EI values, comparable to that at site for nontreated control without occlusion (NCWO), but no statistically significance change was found (Fig. 7c). Comparison of these *in vivo* results with our *in vitro* cytotoxicity assay (Fig. 6) reveals a correlation between the two, which is in line with the results of previous studies (Mehling and Hensen, 2004; Savić et al., 2006; Savic et al., 2007). Overall, the irritancy test results fully support the suitability of investigated vehicles as prospective pharmaceutical bases. Analyzing the TEWL values (Fig. 7a), it is noteworthy a small decrease of TEWL at all treated sites related to corresponding baselines, but again comparable with changes at nontreated sites (NCO and NCWO). However, as we remind of well established fact that skin occlusion *per se* may produce a significant TEWL increase (Levin and Maibach, 2005) than obtained results may additionally support acceptable skin tolerability for investigated vehicles.

The results obtained for skin hydration (SCH) show a significant increase at all treated sites related to the baselines but importantly also to the both nontreated controls (Fig. 7b). The latter leads to the conclusion that occlusion itself did dot contribute to the skin hydration at treated sites. Registered changes, however, were irrespective of used lipophilic excipient.

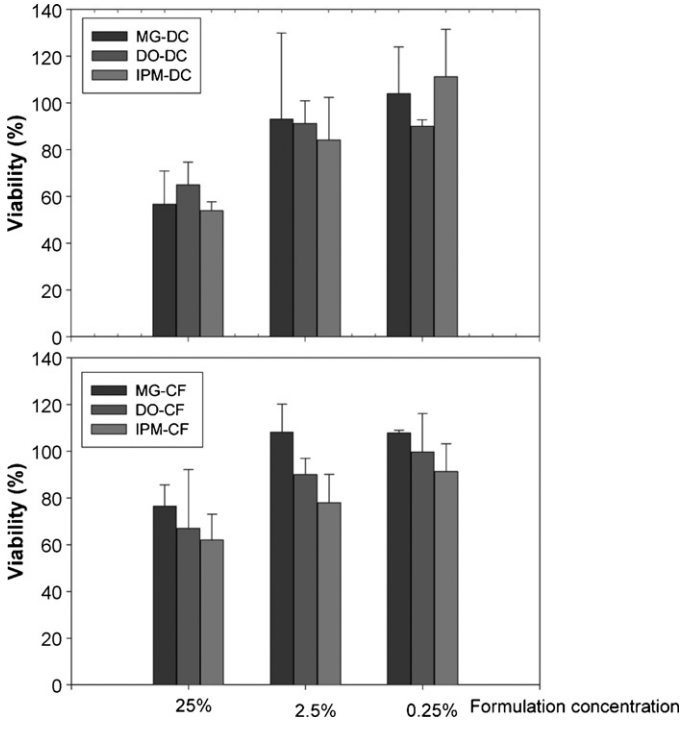


**Fig. 6.** *In vitro* skin irritation test: concentration-viability histograms for (a) DC and (b) CF loaded samples.

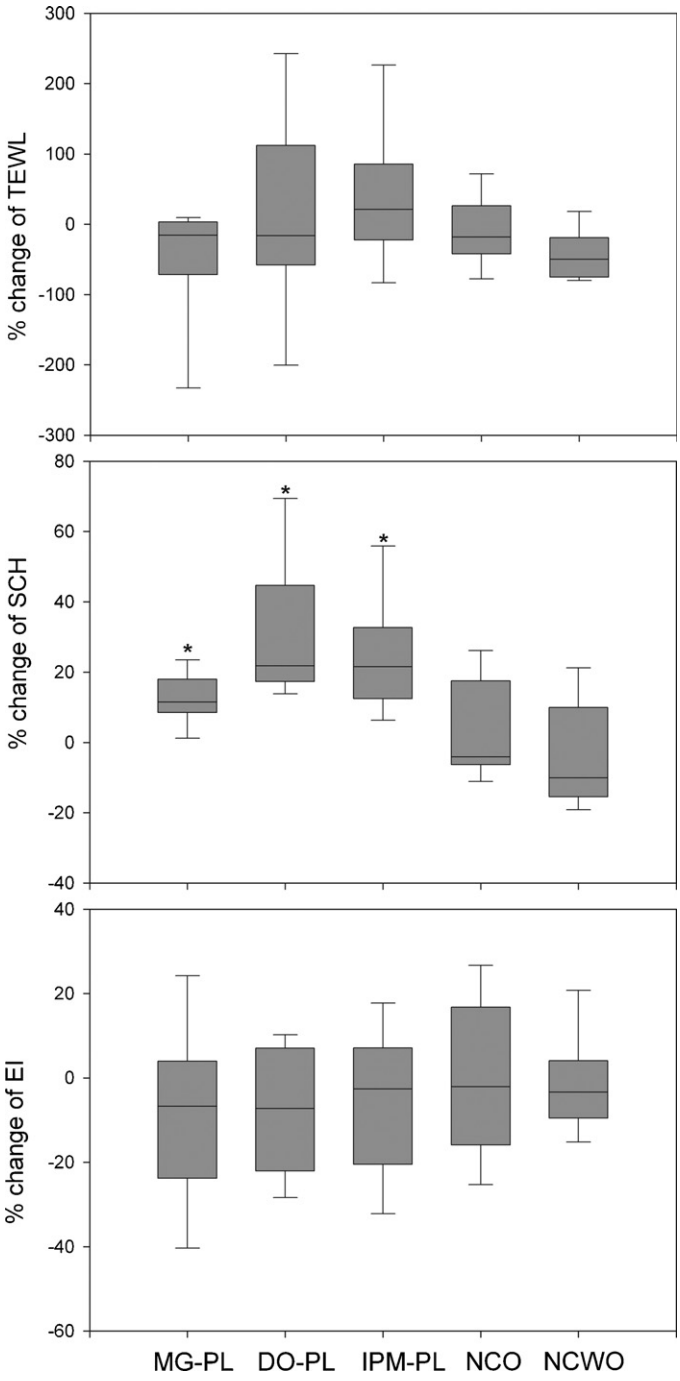


**Fig. 7.** The influence of vehicles (MG-PL, DO-PL and IPM-PL) on *in vivo* measured skin parameters: trans-epidermal water loss (TEWL), skin hydration (SCH) and erythema index (EI). Parameters were expressed as the percentage change on the second vs. first day, and plotted as vertical bars with medians, 25th and 75th percentile (10th and 90th percentile as error bars). The effects of different formulations were compared mutually and related to NCO and NCWO; $^{*}p < 0.05$.

## 4. Conclusion

This study deals with the physicochemical properties and *in vitro*/*in vivo* performance aspects of the emulsion samples based on a novel mixed emulsifier with a surfactant of natural origin,

cetearyl glucoside and cetearyl, containing three different lipophilic excipients and two model drugs of different type.

Distinct variations in the colloidal structure of different vehicles were found, but considering at the same time the presence of the drug. The differences were demonstrated as different rheological profiles accompanied to the certain degree by different modes of water distribution within the structure and were strongly dependent on drug nature: an amphiphilic ionic drug, a salt of a weak acid (DC), against the second one a weak base (CF).

*In vitro* permeation data obtained using ASC membranes in an infinite dose-type of experiment stressed the importance of the vehicle/solute interactions in case of small variation in formulation composition, asserting the drug properties in the first hours of permeation and rheological profile of the vehicles in the later phase of experiment as decisive factors.

*In vitro* skin irritation test demonstrated a mild nature of the emulsifying wax and the absence of negative effects of used oil phases on cell viability in the formulation concentrations correspondent to the therapeutic need. This result alongside with data obtained from in vivo study, could additionally promote usefulness of investigated emulsion systems as prospective "ready to use" pharmaceutical bases for a variety of model drugs.

## References


Al-Bawab, A., Friberg, S.E., 2006. Some pertinent factors in skin care emulsions. Adv. Colloid Interf. Sci. 123–126, 313–322.

Bárány, E., 2000. Human in vivo skin irritancy testing. In: Lodén, M., Maibach, H.I. (Eds.), Dry Skin and Moisturizers. CRC Press LLC, Boca Raton, pp. 243–250.

Bárány, E., Lindberg, M., Lodén, 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. Technol. 3, 126–132.

Clarys, P., Alewaeters, K., Lambrecht, R., Barel, A.O., 2000. Skin color measurements: comparison between three instruments: the Chromameter®, the DermaSpectrometer® and the Mexameter®. Skin Res. Technol. 6, 230–238.

Eccleston, G.M., 1997. Functions of mixed emulsifiers and emulsifying waxes in dermatological lotions and creams. Colloids Surf. A: Physicochem. Eng. Aspects 123/124, 169–182.

Eccleston, G.M., Behan-Martin, M.K., Jones, G.R., Towns-Andrews, E., 2000. Synchrotron X-ray investigations into the lamellar gel phase formed in pharmaceutical creams prepared with cetrimide and fatty alcohols. Int. J. Pharm. 203, 127–139.

Fairhust, C.E., Fuller, S., Gray, J., Holmes, M.C., 1998. Lyotropic surfactant liquid crystals. In: Demus, D., Goodby, J., Gray, G.W., Spiess, H.W., Vill, V. (Eds.), Handbook of Liquid Crystals, vol. 3. Wiley-VCH, Weinheim, pp. 341–392.

Fang, J.Y., Leu, Y.L., Fang, C.L., Chiu, H.C., 2003. In vitro and in vivo evaluations of the efficacy and safety of skin permeation enhancers using flurbiprofen as a model drug. Int. J. Pharm. 255, 153–166.

Fluhr, J.W., Kuss, O., Diepgen, T., Lazzerini, S., Pelosi, A., Gloor, M., Berardesca, E., 2001. Testing for irritation with a multifactorial approach: comparison of eight noninvasive measuring techniques on five different irritation types. Br. J. Dermatol. 145, 696–703.

Freshney, R.I., 1994. Culture of animal cells: A manual of basic technique, 3rd ed. Wiley J. Liss, New York.

Hoffmann, C., Müller-Goymann, C.C., 2005. Use of artificial skin constructs in permeation studies of clindamycin phosphate. Pharmazie 60, 350–353.

Korhonen, M., Lehtonen, J., Hellen, L., Hirvonen, J., Yliruusi, J., 2002. Rheological properties of three component creams containing sorbitan monoesters as surfactants. Int. J. Pharm. 247, 103–114.

Kriwet, K., Müeller-Goymann, C.C., 1993. Binary diclofenac diethylamine–water systems: micelles, vesicles and lyotropic liquid crystals. Eur. J. Pharm. Biopharm. 39, 234–238.

Levin, J., Maibach, H., 2005. The correlation between transepidermal water loss and percutaneous absorption: an overview. J. Control. Release 103, 291–299.

Makai, M., Csanyi, E., Nemeth, Z., Palinkas, J., Erös, I., 2003. Structure and drug release of lamellar liquid crystals containing glycerol. Int. J. Pharm. 256, 95–107.

Mehling, A., Hensen, H., 2004. Comparative studies on the irritation potential of surfactants. Exog. Dermatol. 3, 191–200.

Mondain-Monval, O., 2005. Freeze fracture TEM investigations in liquid crystals. Cur. Opin. Colloid Interf. Sci. 10, 250–255.

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

Nemeth, Z., Halasz, L., Palinkas, J., Bota, A., Horanyi, T., 1998. Rheological behaviour of a lamellar liquid crystalline surfactant–water system. Colloid Surf. A 145, 107–119.

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).

Pena, L.E., Lee, B.L., Stearns, J.F., 1994. Structural rheology of model ointment. Pharm. Res. 11, 875–881.

Ponec, M., 2002. Skin constucts for replacement of skin tissues for in vitro testing. Adv. Drug Del. Rev. 54, S19–S30.

Refai, H., Müller-Goymann, C.C., 2002. The influence of dilution of topical semisolid preparations on hydrocortisone permeation through excised human stratum corneum. Eur. J. Pharm. Biopharm. 54, 143–150.

Robles-Vasquez, O., Corona-Galvan, S., Soltero, J.F.A., Puig, J.E., Tripodi, S.B., Valles, E., Manero, O., 1993. Rheology of lyotropic liquid crystals of Aerosol OT. II. High concentration regime. J. Colloid Interf. Sci. 160, 65–71.

Rogiers, V., 2001. EEMCO guidance for the assessment for transepidermal water loss in cosmetic sciences. Skin Pharmacol. Appl. Skin Physiol. 14, 117–128.

Savić, S., Tamburić, S., Savić, M., Cekić, N., Milić, J., Vuleta, G., 2004. Vehicle-controlled effect of urea on normal and SLS-irritated skin. Int. J. Pharm. 271, 269–280.

Savić, S., Savić, M., Tamburić, S., Vuleta, G., Vesić, S., Müller-Goymann, C., 2007. An alkylpolyglucoside surfactant as a prospective pharmaceutical excipient for topical formulations: the influence of oil polarity on the colloidal structure and hydrocortisone in vitro/in vivo permeation. Eur. J. Pharm. Sci. 30, 441–450.

Savić, S.D., Savić, M.M., Vesić, S.A., Vuleta, G.M., Müller-Goymann, C.C., 2006. Vehicles based on a sugar surfactant: colloidal structure and its impact on in vitro/in vivo hydrocortisone permeation. Int. J. Pharm. 320, 86–95.

Schäfer-Korting, M., et al., 2008. The use of reconstructed human epidermis for skin absorption testing: results o the validation study. ATLA 36, 161–187.

Schmook, F.P., Meingassner, J.G., Billich, A., 2001. Comparison of human skin or epidermis models with human and animal skin in in-vitro percutaneous absorption. Int. J. Pharm. 215, 51–56.

Smith, E.W., Surber, C., Tassopoulos, T., Maibach, H., 2002. Topical dermatological vehicles: a holistic approach. In: Bronaugh, R.L., Maibach, H.I. (Eds.), Topical Absorption of Dermatological Products. Marcel Dekker, New York, pp. 457–463.

Spielmann, H., et al., 2007. The ECVAM international validation study on in vitro tests for acute skin irritation: report on the validity of the EPISKIN and EpiDerm assays and on the skin integrity function test. ATLA 35, 559–601.

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

Williams, A.C., Barry, B.B., 2004. Penetration enhancers. Adv. Drug Deliv. Rev. 56, 603–618.

Winkler, A., Müller-Goymann, C.C., 2002. Comparative permeation studies for δ-aminolevulinic acid and its n-butyl ester through stratum corneum and artificial skin constructs. Eur. J. Pharm. Biopharm. 53, 281–287.