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Research paper

Compounding of a topical drug with prospective natural surfactant-stabilized pharmaceutical bases: Physicochemical and *in vitro*/*in vivo* characterization – A ketoprofen case study

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ABSTRACT

Recently, healthcare professionals again began realizing the benefits of preparing customized medications to meet specific patient needs. The objective of this work was to develop and evaluate simple pharmaceutical bases stabilized with natural-origin surfactant of alkyl polyglucoside (APG) type as prospective ready-to-use bases and compare them to widely used pharmacopoeial ones. Additionally, the ability of the formulated bases to sustain isopropyl alcohol was assessed as well as its influence on ketoprofen skin absorption (as a co-solvent and potential penetration enhancer). In order to evaluate the manifold characteristics a topical drug product should possess, a comprehensive characterization was performed using different techniques.

Physicochemical characterization demonstrated satisfactory physical stability of APG-stabilized bases upon the addition of alcohol. *In vitro* release/permeation studies failed to show significant difference in ketoprofen liberation/permeation profiles from different bases. However, the extent of ketoprofen delivery *in vivo* was clearly increased from APG bases, relative to that obtained from pharmacopoeia quality one, implying a distinct influence of the emulsion systems' colloidal structures. Taking also into account the rheological behavior of APG bases, revealing their ameliorated sensory characteristics, it could be concluded that the investigated APG bases could be considered as preferential option in drug compounding related to the conventional ones.

1. Introduction

Compounding or extemporaneous dispensing is usually defined as small-scale manufacturing of medicines from basic ingredients and has been considered obsolete by many [1]. However, in the 1990s, healthcare professionals again began realizing the benefits of preparing customized medications to meet specific patient needs. Additionally, in some countries, extemporaneous preparations supplied by both community and hospital pharmacies are still in high demand. The issue of concepts and trends in pharmaceutical compounding is one of the compulsory topics regularly considered by pharmaceutical organisations worldwide. In spite of all the technological advances made by pharmaceutical companies in developing new drug products, these tailored medicines remain significant, especially in dermatology [2]. Bases commonly used as vehicles for dermatological preparations are stabilized by traditional anionic and non-ionic surfactants. However, for some time, in the eyes of both pharmacists and their patients, many of the conventionally used bases are considered esthetically unsuitable and hence are applied unwillingly [3].

On the other hand, constant promotion of natural-origin products by the cosmetic industry has led to corresponding demands of a modern patient, and the pharmaceutical practice has to live up to these expectations, especially concerning topical dosage forms [4]. It is a well-known fact that the use of traditional surfactants plays a critical role in current exposure situation and gives rise to environmental concern, since they are readily reaching the environment from consumer products via sewage and industrial discharges [5]. Therefore, so-called 'green surfactants' based on natural raw materials have received increasing attention [6].

Alkyl polyglucoside (APG) emulsifiers are natural-origin surfactants initially described more than 100 years ago. Nevertheless, they were hastily disregarded until the 1980s, when large-scale

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cost-efficient production was achieved [7,8]. APGs are prepared from natural renewable raw materials, natural sugar unit and natural or non-natural fatty alcohol. However, their main attractiveness lies in highly favorable environmental profile (rapid biodegradation and low toxicity) and complimentary dermatological properties [9,10].

In this context, we were interested to develop and evaluate simple APG-stabilized pharmaceutical bases of emulsion type as prospective ready-to-use vehicles and compare them to widely used conventional (pharmacopoeial) ones. Investigated oil in water (o/w) creams were based on natural-origin mixed non-ionic emulsifier comprised of cetearyl glucoside and cetearyl alcohol. A comprehensive characterization was performed both with and without the addition of selected active pharmaceutical ingredient (API).

Ketoprofen was chosen as a model non-steroidal anti-inflammatory drug (NSAID) frequently used for the treatment of rheumatoid arthritis, osteoarthritis, ankylosing spondylitis and similar highly prevalent disorders. It is considered equally or more potent than other NSAIDs [11,12].

It is universally accepted that the vehicle in which the active ingredient is delivered can affect the skin penetration depth and absorption rate into the epidermis [13,14]. The penetration of topical modalities is often limited by the stratum corneum itself [15,16]. Therefore, in order to improve ketoprofen cutaneous delivery from emulsion systems, several strategies can be considered as follows: chemical penetration enhancers' addition, use of novel vehicle systems or physical enhancement methods (iontophoresis, sonophoresis, electroporation, etc.) [16,17]. Due to the fact that many APIs that are frequently incorporated into pharmaceutical bases *ex tempore* are characterized with poor aqueous solubility (ketoprofen being such an example), the influence of isopropyl alcohol (IPA) addition to investigated bases was also assessed. To our knowledge, there is no available data on the influence of isopropyl alcohol addition, as a co-solvent and potential penetration enhancer, to APG-based emulsion vehicles.

In order to evaluate the manifold characteristics a topical drug product should possess, physicochemical, *in vitro*/*in vivo* biopharmaceutical and safety considerations were assessed. The influence of IPA addition on both drug solubility and physical stability of investigated emulsion bases was evaluated by polarization microscopy. A further insight into stability was gained by physicochemical characterization of investigated vs. referent samples through rheological measurements and thermal techniques. The potential of the designed APG-stabilized bases as drug delivery systems was assessed through several *in vitro*/*in vivo* methods, taking into consideration that (with the exception of topical glucocorticoids) no technique has been universally accepted as self-sufficient to fully characterize penetration/permeation profiles of topical drugs [18]. The rational for the inclusion of alcohol to semisolids is its expected interaction with stratum corneum towards decreasing the barrier function and allowing enhanced drug penetration. However desirable this penetration enhancement may be, one should not neglect the possible irritation that could be exerted on the skin. Therefore, the influence of the very base in which IPA is to be incorporated sometimes may determine whether or not some side effects may be expressed. These safety considerations were tested through *in vitro* cytotoxicity assay and *in vivo* skin irritation/performance study.

2. Materials and methods

2.1. Materials

Test samples (labelled S68) were stabilized with mixed non-ionic alkyl polyglucoside emulsifier (cetearyl glucoside and cetearyl alcohol, Sepineo SE® 68, kindly donated by Seppic, Paris, France). A preliminary formulation study showed that the emulsifier concentration used (8% w/w) allowed the formation of creams with satisfactory organoleptic characteristics, both prior to and upon the addition of various co-solvents (data not shown). Pure cetostearyl alcohol was added as a co-stabilizer. All creams were prepared with medium chain triglycerides (Saboderm TCC, Sabo S.R.L., Levate, Italy) and preserved with Euxyl® K300 (Schülke&Mayr, Norderstedt, Germany).

Polysorbate 60 used for the preparation of referent pharmacopeial vehicle (labelled N), Non-ionic hydrophilic cream (DAB 2006) (Tween® 60), was purchased from Merck KGaA, Darmstadt, Germany [19].

Both test and reference samples were prepared with (samples S68ipa, Nipa) and without (S68, N) the addition of isopropyl alcohol (10% (w/w), Brenntag, Wien, Austria). Detailed composition of investigated samples is given in Table 1. Upon the addition of ketoprofen 2.5% (w/w) (Merck KGaA, Darmstadt, Germany), active samples were labelled as follows: S68-K, S68ipa-K, N-K and Nipa-K. Double distilled water was used for the preparation of all samples. Compounds used were of pharmacopoeial quality (Ph. Eur. 6.0), whenever possible.

D-squame® skin sampling discs (CuDerm, Dallas, USA) were used for tape stripping of human stratum corneum (SC) with its fully cured medical grade synthetic polyacrylate ester adhesive.

2.2. Methods

2.2.1. Preparation of samples

Samples stabilized with APG emulsifier were prepared according to previously standardized procedure [20]. Emulsifier was heated with the oily phase at 70 °C and then added to the water phase at the same temperature, followed with precisely defined mixing dynamic optimized by Savic et al. [20]. The addition of IPA and preservative was performed dropwise upon cooling the cream below 35 °C. Ketoprofen was suspended in prepared vehicles in order to evaluate the vehicles' potential to dissolve the model drug. Referent Non-ionic hydrophilic cream was prepared according to DAB 2006 [19].

Prepared samples were allowed 7 days equilibration before being submitted to selected characterization techniques.

2.2.2. pH and conductivity measurements

pH measurements were taken by direct immersion of pH meter glass electrode (Hanna instruments HI 9321, Michigan, USA) in investigated samples. In order to assess both the emulsion type (mode of water distribution) and sample stability, conductivity measurements were performed (conductivity meter CDM 230, Radiometer, Brønshøj, Denmark). All measurements were performed initially (a week after preparation), after 30 and 90 days storage at room temperature.

Table 1
Composition of the investigated samples with and without isopropyl alcohol as the selected co-solvent/potential penetration enhancer.

% (w/w)	S68	S68ipa	N	Nipa
Cetearylglucoside and cetearyl alcohol	8.0	8.0	–	–
Cetostearyl alcohol	1.0	1.0	10.0	10.0
Medium chain triglycerides	10.0	10.0	–	–
Preservative	0.5	0.5	0.5	0.5
Isopropyl alcohol	–	10.0	–	10.0
Polisorbate 60	–	–	5.0	5.0
White soft paraffin	–	–	25.0	25.0
Glycerol, 85%	–	–	10.0	10.0
Water, double distilled to	100	100	100	100

2.2.3. Polarization microscopy

The first insight into the colloidal structure of the samples was screened by a Carl Zeiss ApoTome Imager Z1 microscope (Zeiss, Göttingen, Germany) integrated with digital AxioCam ICc1 camera and AxioVision 4.6 computer software. A pin-tip amount of each sample was taken from three separate sites within the sample and smeared on the microscope glass slide, covered with the cover slip and pressed to make it as thin as possible. Magnifications 200× and 400× were captured with cross-polarizer in bright field using wavelength (λ) plate, to detect birefringence. The aim of comparative assessment of polarization micrographs was to evaluate difference in test and referent bases' microstructure, behavior upon IPA addition, as well as for descriptive assessment of IPA's contribution to ketoprofen solubility.

2.2.4. Rheological measurements

The rheological characterization was conducted in order to evaluate the preliminary physical stability, as well as to screen colloidal structure and changes induced within by the addition of alcohol phase to both types of investigated bases in a predetermined period of time.

The continuous flow behavior was assessed to evaluate the preliminary physical stability of colloidal systems. Additionally, flow and viscoelastic properties are known to exhibit influence on both drug release from semisolids and sensory properties crucial for patient acceptability [21].

Continual measurements were performed for both placebo and active samples, initially (after 7 days) and after 90 days storage at room temperature (Rheometer Rheolab MC 120, Paar Physica, Ostfildern, Germany). All measurements were carried out using cone/plate measuring system (diameter 50 mm, angle 1°), with 0.05 mm sample thickness, at 20 ± 0.1 °C (in triplicate). During continual testing, controlled shear rate procedure was applied (shear rate 0–200 s^{-1} and back again to the start point, each stage lasting 120 s). Assessed parameters were flow behavior, yield value and hysteresis loop. In order for yield stress to be determined, flow curves obtained in controlled shear rate procedure (shear rate 0–30 s^{-1}) were analyzed using the accompanying Physica US200 software.

2.2.5. Differential scanning calorimetry (DSC)

DSC measurements were performed in order to compare phase transitions of investigated bases. Obtained DSC scans were expected to provide additional information concerning colloidal structure of the assessed samples, both prior to and upon alcohol and/or drug addition.

Small amounts of samples (between 10 and 12 mg) were accurately balanced (XP205 DeltaRange® analytical balance, Mettler Toledo, Giessen, Germany) in aluminum pans, hermetically sealed and analyzed using Mettler DSC 820 (Mettler Toledo, Giessen, Germany). As a reference, an empty pan was used. Samples were heated from ambient temperature (25 °C) to 105 °C, with the heating rate of 2 K/min to compare endothermic transition enthalpies of test and reference samples. Duplicate measurements, at least, were taken for all samples.

2.2.6. Thermogravimetric analysis (TGA)

TGA was performed in order to assess the mode of water distribution in investigated samples, possibly implying the complexity of their colloidal structure and the potential to additionally hydrate the skin.

Small amount of samples were placed in open aluminum pans and heated in Netzsch STA 409PG (Netzsch, Selb, Germany), from 30 to 110 °C with isothermal segments at the beginning and ending of each measurement. The heating rate was set at 5 K/min. All samples were analyzed in duplicate.

2.2.7. In vitro permeation studies through synthetic membranes, skin model membranes and isolated stratum corneum

Preliminary screening of ketoprofen release profiles was performed through inert synthetic membranes. Each sample (2 g) was carefully placed in VanKel Enhancer Cells® (VanKel Industries Inc., CA, USA) providing no air gaps were inserted. Prepared diffusion cells were placed in apparatus for dissolution testing equipped with mini paddle system (Erweka DT 600, Heusenstamm, Germany). Drug release was monitored for 6 h in 300 ml of receptor medium (phosphate buffer solution pH 7.4, PBS, USP30). Donor and acceptor phases were separated by previously rehydrated cellulose acetate membrane. Constant temperature (32 ± 0.5 °C) and paddle rotation speed (50 rpm) were maintained throughout the experiment. In order to retain sink conditions, aliquots (5 ml) were withdrawn from the acceptor compartment at specified time intervals and immediately replaced with fresh PBS. Samples were filtered and analyzed for ketoprofen spectrophotometrically (Evolution 300, Thermo Fisher Scientific, LE, UK), at 260 nm. *In vitro* release study was performed in triplicate for all active samples.

Ketoprofen liberation kinetic was assessed by mathematical modelling through four models befitting for semisolids: zero-order, first-order, Higuchi and Hixon-Crowell kinetics.

Consecutively, *in vitro* permeation was assessed through artificial skin constructs mounted on Franz cells, this being the recommended release testing method for semisolid preparations. Artificial skin constructs (ASCs) were cultivated and prepared according to the previously described procedure [22,23].

Due to the known lack of diffusion resistance of ASCs [24], permeation experiments were conducted with isolated stratum corneum (SC) as well. SC was isolated from an abdomen part of a donor (female, 37 year old) after a plastic surgery. Isolation was performed using an aqueous trypsin solution of 0.5 mg/mL (Carl Roth GmbH, Karlsruhe, Germany). For this purpose, the fatty tissue was first removed from the skin samples after which the skin was transferred, dermal side down, into a Petri dish containing the trypsin solution. After an incubation time of 48 h at 37 °C, the SC could be removed carefully with forceps. The isolated SC pieces were washed once in an aqueous trypsin-inhibitor solution (0.4 mg/mL) type II-O: chicken egg white (Sigma–Aldrich Chemie GmbH, Steinheim, Germany) followed by three times washing with distilled water. SC was stored in a desiccator until use.

Both *in vitro* permeation studies were carried out with modified Franz cells ($n = 6$). The donor compartment was filled with the sample (infinite dose; permeation area 0.418–0.494 cm^2), whereas the receiver compartment (volume: 5.21–6.33 ml) contained PBS, which was constantly stirred with a rotating magnet at 300 rpm. The acceptor compartments were mounted in a water bath at 37 °C, while the temperature of membranes was 32 ± 0.1 °C. Aliquots of 250 μl were taken over 30 h, and ketoprofen assay was performed by high performance liquid chromatography (HPLC, Waters, Eschborn, Germany). Each aliquot was replaced with the same amount of fresh buffer to maintain sink conditions. Cumulative amounts of drug penetrating per unit area (μg/cm^2) were plotted against time. In order to assess the thermodynamic activity of the model drug and its influence on permeation studies, concentrations of saturation (Cs) of ketoprofen in investigated bases were assessed according to Savic et al. [25].

2.2.8. In vivo skin absorption assessment – tape stripping method

Stripping technique of human stratum corneum was used in order to compare ketoprofen skin bioavailability from APG-based and reference samples. Additionally, it was of interest to determine whether and to what extent IPA addition enhanced ketoprofen penetration.

Formulations were tested on the volar aspect of a forearm of 6 healthy human volunteers (5 females and 1 male, aged 24–29).

Written informed consent was obtained from each volunteer before the study commenced. The protocol of the study followed the recommended guidelines set by the Declaration of Helsinki and was approved by the local Ethical Committee. A dose of 5 mg/cm^2 was uniformly applied onto the assigned skin sites previously delimited using a permanent marker pen. Test samples were left in contact with the skin for 2 h.

Twelve adhesive D-squame® tapes with constant surface area (3.8 cm^2) were used to successively remove superficial SC layers, after being submitted to uniform pressure (140 g/cm^2) for 10 s. Simultaneously, transepidermal water loss (TEWL) was evaluated using Tewameter®TM210 (Courage + Khazaka, Köln, Germany), prior to stripping (at baseline), after 7th and 12th tape stripped, due to normalization of individual skin thickness and as a measure of skin barrier condition. Each tape was weighted on a high-precision analytical balance (Sartorius BP210D, Goettingen, Germany) prior to and immediately after the procedure in order to quantify removed SC layers. A pair of forceps was used while manipulating the tapes, to exclude contamination with corneocytes of the researcher. The first two tapes were discarded, and the following 10 tapes were each submitted to the following extraction procedure. Each tape strip was carefully placed into a glass centrifuge tube, and 5 ml of ethanol (70% v/v) was added. Tubes were submitted to sonification (Sonorex RK102H, Bandelin, Berlin, Germany) for 15 min and subsequently centrifuged at 4000 rpm (equivalent to 1789 g acceleration) for 5 min (Tehtnica, Zelezniki, Slovenia). Obtained supernatants were analyzed for ketoprofen content with HPLC.

The weight of each tape was used to calculate the thickness of removed SC layer according to the Eq. (1) [21,26]:

$$T = d/a \cdot \rho \quad (1)$$

where T is the thickness of removed SC layer (μm), d is the difference in the weight of tapes after and prior to the stripping procedure (μg), a is the stripping area (μm^2) and ρ is the approximation of SC density (set at $\rho = 10^{-6}$ μg/μm^3) [27,28].

2.2.9. HPLC

Data were collected and analyzed using HPLC system Agilent series1100 (CA, USA) with DA detector and quaternary pump. Separations were performed on a Luna C_{18}, 100 A, 250 mm × 4.6 mm, 5 μm particle column. The sample volume of 20 μl was introduced through a partial loop. The flow rate of the mobile phases was 1.5 ml/min, and the column temperature was set at 25 °C. UV detection was performed at 254 nm. The mobile phase used consisted of acetonitrile/methanol/0.5% w/v ammonium acetate (pH = 5.9) (27/18/55). pH was adjusted with nitric acid (10% v/v). Constructed calibration curves were linear in the investigated range (r = 0.9996), while limits of detection (LOD) and quantification (LOQ) were 3.18 and 10.61 ng/ml, respectively.

2.2.10. In vitro skin irritation test – a cytotoxicity assay

In order to evaluate the safety aspect of a novel APG-stabilized cream aiming to be a potential ready-to-use pharmaceutical base, its effect on skin irritation was initially assessed *in vitro*. It was of substantial interest to consider the ability of the base to sustain the expected IPA's aggressive influence on the skin, added as a co-solvent and potential penetration enhancer.

A modified version of Mosman's MTT reduction method [29,30] was used: ASCs were placed into a 12-well plate containing three different concentrations of test formulations (0.25% (w/w), 2.5% (w/w) and 25% (w/w)) dispersed in Krebs–Ringer buffer (KRB). ASCs placed into pure KRB, and KRB with sodium dodecyl sulfate (SDS, 1% w/w) served as negative and positive controls, respectively. Non-specific MTT reduction by any of the formulation constituents was excluded i.e. was lower than 30% relative to the negative control [31].

After 2 h of incubation, ASCs were rinsed with KRB and transferred into a 24-well plate containing 900 μl of minimally supplemented basal medium (MSBM) and 100 μl aqueous MTT solution (0.5%) in each well, and incubated at 37 °C and 5% CO_2 atmosphere for 2 h. The supernatant was then removed, and the dyed ASCs were discolored in 500 μl lysis solution containing SDS (2.73 g), hydrochloric acid (32.5%; 3.64 g), water (88.18 g) and IPA (905.45 g). The colored solution (150 μl) was 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 irritation/performance study

Following the results obtained by previously described *in vitro* cytotoxicity test, the investigated (S68, S68ipa) vs. reference placebo bases (N, Nipa) were screened for the general skin performance *in vivo* as well.

In vivo assessment of placebo samples' irritation potential was conducted by non-invasive bioengineering techniques. Ten female volunteers (mean age, 24.9 ± 1.8) participated in a 24-h study under occlusion. Prior to the study, volunteers were thoroughly informed about possible treatment effects, as well as expected conduct during the experiment. Signed written consents were obtained in accordance with the Helsinki Declaration and local Ethical Committee. Volunteers were obliged to refrain from using topical products 2 days prior to and throughout the experiment.

Volunteers were instructed to spend 30 min in the study room prior to measurements in order to adapt to room conditions (temperature 23 ± 1 °C, relative humidity 35 ± 5%), after which initial measurements were taken. The following parameters were evaluated: SC hydration (SCH), TEWL, skin pH and erythema index (EI). All measurements were conducted on flexor aspects of forearms, at 4 × 4 cm square application sites. A plastic template was applied to delimit test areas that were placed 1–2 cm apart and 6 cm away from the wrist and elbow. A site per each arm was reserved for non-treated control under occlusion (NCO) and without occlusion (NC).

After initial values were obtained, placebo samples were applied in quantities of 15 mg/cm^2 and covered with silicone film (Parafilm®, USA) and fixed with hypoallergenic adhesive tapes (3 M™Transpore™ tape, 3M Health Care, Bracknell, UK). One hour upon removal of the 24-h occlusion, all parameters were reassessed. Measurements were performed with set of Courage + Khazaka equipment (Köln, Germany): Cutometer® MPA 580 for pH and SCH evaluation, Mexameter® MX18 and Tewameter® TM210, for EI and TEWL assessments, respectively. All parameters were measured according to valid guidelines and documents [32–34].

2.2.12. Statistical analysis

Results were presented as mean values ± SD. Statistical analysis was carried out using Student's t-test and analysis of variance using SigmaStat 3.11 (Systat, CA, USA), depending on the nature of the data ($p < 0.05$). Parameters of *in vivo* measurements were assessed with a one-way ANOVA followed by Tukey *post hoc* test, whenever appropriate.

3. Results and discussion

3.1. Physicochemical characterization

When evaluating a novel pharmaceutical excipient for dermatological preparations, various criteria must be complied. It is always of interest to develop topical dosage forms that will provide satisfactory stability, cutaneous tolerability, as well as consistent drug

levels at the application site. Therefore, a comprehensive physicochemical characterization is an imperative.

Performed characterization through polarization microscopy has confirmed that emulsion bases stabilized with cetearyl glucoside and cetearyl alcohol mixture are characterized by distorted Maltese crosses when observed between crossed polars (Fig. 1) [20,23], suggesting lamellar liquid crystalline phase formation. In addition, lamellar gel network could be seen as complex layers surrounding larger droplets or floccules of smaller ones [20].

In general, emulsions containing non-ionic surfactants are known to reach equilibrium in several weeks [35]. Therefore, polarization micrographs were recorded 7 and 30 days after preparation, in order to obtain deeper insight into specific colloidal structure. In parallel, it was of interest to descriptively evaluate the potential of both IPA addition and base itself to dissolve initially suspended drug (Fig. 2).

It could be clearly observed that fairly large and irregularly shaped droplets dominate the structure of IPA-loaded samples (Fig. 1b and d). Evidently, this is the result of alcohol addition since it is implemented mainly in the interfacial layer, resulting in transition from small globules to short cylinders and finally large cylindrical structures [36].

The structure of reference samples (N and Nipa) could be observed as relatively thick, matrix-like system, rather than with isolated dispersed droplets. Apart from the fact that the content of water is lower (approximately 50% in N and 40% in Nipa) in comparison with APG-based samples (80% or 70%, in S68 and S68ipa, respectively), it is likely that the surplus of cetostearyl alcohol (10% w/w, in N-labelled bases) forms the lipophilic gel phase, which immobilizes the dispersed phase consisting mainly of white petrolatum [37].

By closely examining recorded micrographs of drug-loaded samples (Fig. 2), a certain improvement in ketoprofen solubility could be observed in sample S68-K after 1-month storage, indicating that APG-stabilized base exerts certain potential concerning ketoprofen dissolution. As for IPA-loaded samples (S68ipa-K and Nipa-K), the solubility enhancement was even more obvious. It is hard to say whether the same trend applies to the reference sample (N-K) as well, since large clusters of suspended drug could still be observed.

Taking into account the rheological measurements, all samples exhibited shear-thinning flow behavior with moderate (S68, S68-K, S68ipa, Nipa, N-K and Nipa-K) to pronounced (S68ipa-K and N) thixotropy, which is considered a property desirable for topically applied preparations. Flow curves show (Fig. 3a) that incorporation of IPA does not alter the structure of APG-stabilized bases. However, the same could not be stated for the reference base since changes in flow curve and hysteresis pronounced decrease indicate vehicle–IPA interaction. Upon ketoprofen incorporation into APG samples yield stress values were increased (Table 2), as expected, considering the fact that ketoprofen is suspended in investigated bases. Changes in flow behavior of S68ipa-K (Fig. 3b) could be explained as a result of ketoprofen's solubility in IPA. The dissolved fraction could allow further drug-vehicle interaction leading to complex structuring and subsequent sample thickening. The thickening should partially be attributed to IPA evaporation during the measurements. Samples S68-K, S68ipa-K and N-K after 3 months did not show significant changes in rheological profiles (data not shown). The change in flow properties of Nipa-K (presence of hysteresis loop) can be interlinked with the dissatisfying preliminary physicochemical stability as well as unsatisfactory applicative characteristics.

Continual rheological measurements indicate preferential physicochemical stability of APG bases, especially regarding IPA loading potential, when compared to the reference ones.

DSC measurements were assessed in order to compare phase transitions of investigated bases, both in the light of different colloidal structures (revealed by polarization microscopy and rheological measurements) and alcohol phase addition. Obtained thermoanalytical characteristics revealed substantial difference in APG and reference bases, reflected through the shape of the curves, the peak temperatures and the total enthalpies of the melting process. APG bases were characterized by a single endothermic peak at 80–85 °C, relatively sharp in comparison with the ones assigned to reference samples (N and its modification with IPA – Nipa). Scan of reference samples showed broad endotherm peaking at 70–85 °C, with a broad shoulder at 57.4 °C, in case of Nipa sample. Such endotherms are often difficult to interpret. However, when considered in conjunction with the rheological data [38], they confirm the overall difference in the two bases' microstructure.

The alcohol addition seems to induce a subtle shift of the curves towards both lower ΔH and peak temperature values (ΔH 1708.6 and 1595.6 mJ/mg, for S68 and S68ipa, respectively), with the exception of Nipa-K sample where the difference is negligible. The presence of ketoprofen did not induce a notable change in assessed parameters since the melting enthalpy was not considerably altered, especially when comparing drug-loaded samples to the corresponding ones with IPA (Table 3). Therefore, although DSC measurements were assessed in hope of acquiring additional information of the amount of drug being solubilized or dissolved within the system [39], no assumptions of the sort could be made, presumably because of low ketoprofen concentration in investigated creams (usual therapeutic concentration of 2.5% w/w). The considerable activation energy required to melt APG-based samples may be interpreted by the formation of complex lamellar phases (both liquid crystalline and gel network remnants) emphasizing even more the intrinsic difference in bases' microstructure in comparison with reference ones.

To additionally characterize the colloidal structure of the two investigated vehicle types prior to and after alcohol addition, TGA was conducted as a thermal analysis technique frequently employed along with DSC. Obtained DSC results correlate well with thermogravimetric measurements, assessed in order to investigate the mode of water distribution within the system and, hence, gain a deeper insight into the nature of the colloidal structure. Results of TG analysis were acquired both in direct assessment of TGA profiles, which enabled insight into partial weight loss over different temperature ranges, and indirectly through comparison of first

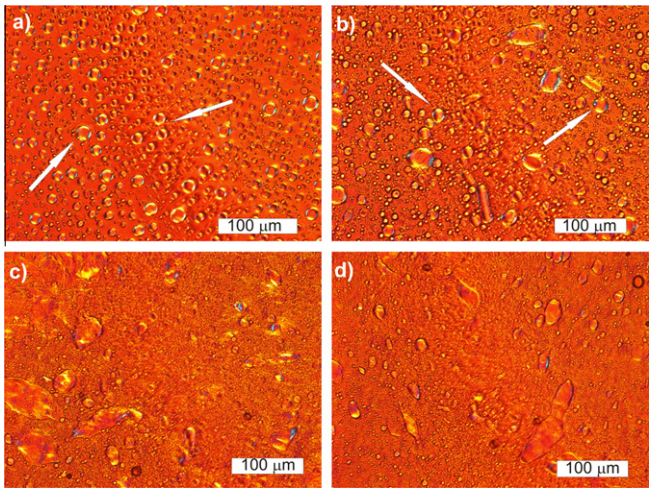

Fig. 1. Representative polarization micrographs of placebo samples of both vehicle type 7 days after preparation: (a) S68, 200×; (b) S68ipa, 200×; (c) N, 200×; (d) Nipa, 200×; bar 100 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

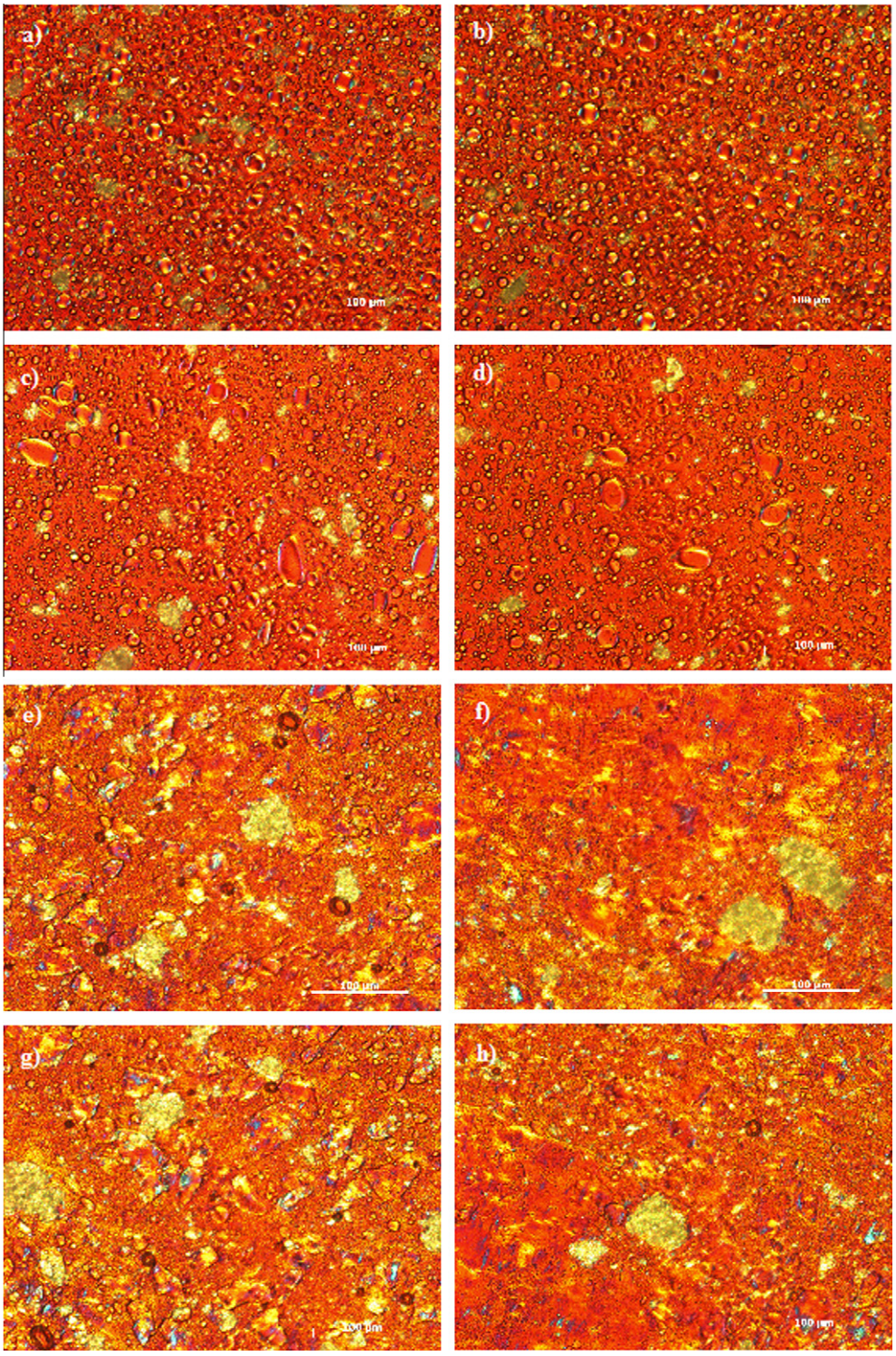

Fig. 2. Micrographs of active samples after 7 and 30 days storage (labelled d7 and d30, respectively): (a) S68-K, d7; (b) S68-K, d30; (c) S68ipa-K, d7; (d) S68ipa-K, d30; (e) N-K, d7; (f) N-K, d30; (g) Nipa-K, d7; (h) Nipa-K, d30; bar 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

derivative TGA curves (DTG profiles) for the evaluation of evaporation rate (Fig. 4).

Although it is often approximated that the observed losses correspond to the water in the system, the loss of other volatile non-aqueous components should not be neglected [40], IPA being such a component in some of the evaluated samples. Results indicate that in sample S68 water is predominantly incorporated as bulk water and hence mainly evaporated at third temperature range (70–110 °C) (Table 3). This probably occurs upon the disruption of the lamellar phases. Furthermore, the surplus of cetostearyl alcohol may be responsible for bounding water as a semihydrate to its long chains [37,40].

Contrary to that, in IPA-loaded sample (S68ipa) and reference bases (N and Nipa), the highest mass loss was observed when

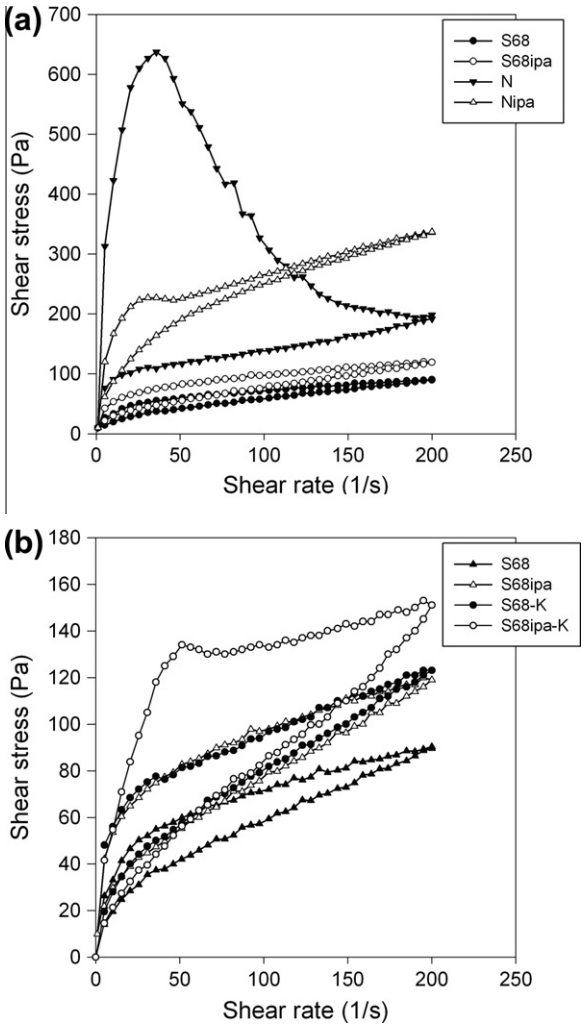

Fig. 3. Continual rheological assessment of the two base types: (a) flow curves of placebo samples showing the influence of the added co-solvent on flow behavior; (b) comparison of placebo (triangle) and active (circle) APG samples' flow curves, with and without the incorporated drug.

Table 2
Yield values of investigated samples (mean ± SD, $n = 3$).

Sample	Yield stress (Pa) ± SD
S68	5.72 ± 0.86
S68ipa	7.45 ± 1.02
S68-K	48.32 ± 2.11
S68ipa-K	34.66 ± 1.97
N	6.65 ± 0.53
Nipa	10.33 ± 0.98
N-K	54.31 ± 2.01
Nipa-K	46.07 ± 1.99

heated from 50 to 70 °C. This is commonly referred to as 'secondary' water. Furthermore, it should be noted that N bases comprise of greater amount of non-volatile components, which influences high residual mass values. As for drug-loaded samples, comparable trends of weight loss could be observed.

One could hypothesize that IPA addition may contribute to the free surface evaporation of corresponding samples. Nevertheless, it is more likely that it evaporates in the second temperature range, implying its firm incorporation into the interfacial layer, previously noticed by the examination of polarization micrographs [36].

By close examination of DTG curves (Fig. 4), it could be observed that the rate of evaporation in sample S68 increases until 74.6 °C, while the corresponding S68ipa curve is shifted towards lower temperatures (64.4 °C). This is an obvious influence of alcohol addition. Small peaks in N and Nipa DTG curves may be the result of cetostearyl alcohol melting, taking into consideration the amount present in N-labelled samples. This is in agreement with results presented by Nesseem [41] and the fact that cetearyl alcohol melting point is around 56 °C.

As for the pH measurements, all the obtained values were within the recommended limits for topical preparations (Table 4) and remained similar throughout the 3-month observation period. As expected, specific conductivity gradually decreased in time, indicating greater structuring of these multi-phase emulsion systems [42]. This decrease is particularly distinct in APG-stabilized bases, confirming the complexity of their microstructure. Additionally, the presence of ketoprofen induced higher conductivity in these samples, which may be interpreted as a contribution of the dissolved fraction of this weak acid. The same could not be stated for samples N-K and Nipa-K, when their conductivity is compared to the corresponding N and Nipa vehicles. This is in accordance with previously argued ability of the investigated bases to dissolve the drug.

3.2. Biopharmaceutical characterization

Biopharmaceutical characterization was conducted through several techniques as it is universally accepted that no single test procedure could provide sufficient results [18]. *In vitro* screening of ketoprofen liberation profiles showed no statistical difference in the amount of drug released from the evaluated samples. When the amount of drug released is plotted vs. the square root of time, a linear relationship is obtained without initial lag time (Fig. 5a). Furthermore, when the obtained profiles were fitted through several kinetic models (zero-order, first-order, Higuchi and Hixon-Crowell), it was observed that release kinetics was best described by the Higuchi principle ($r > 0.99$), indicating that the release is governed via diffusion as a rate-controlling step [43].

In spite of the lack of statistical difference ($p > 0.05$), there is a trend of enhancement in ketoprofen release from sample S68ipa-K starting from the second hour of the experiment, possibly due to a change in ketoprofen thermodynamic activity upon IPA addition. Therefore, in order to obtain a deeper insight into the actual influence of the added co-solvent on APG-stabilized microstruc-

Table 3
Thermoanalytical parameters assessed by TGA and DSC measurements: percentage of partial weight loss over specified temperature ranges and DSC integration data – melting enthalpies, expressed as mean ± SD.

Sample	30–50 °C (%)	50–70 °C (%)	70–110 °C (%)	Residual mass (%)	ΔH (mJ/mg)	Peak temperature of DSC scan (°C)
S68	9.70 ± 1.07	19.47 ± 1.80	41.48 ± 3.12	27.42 ± 4.70	1708.6 ± 93.0	86.1
S68ipa	11.77 ± 0.88	59.58 ± 0.67	3.79 ± 1.11	27.22 ± 0.27	1595.6 ± 110.0	80.8
N	4.57 ± 0.41	30.56 ± 0.88	8.20 ± 0.40	52.24 ± 1.48	948.2 ± 72.1	78.6
Nipa	3.99 ± 0.62	33.67 ± 2.20	8.76 ± 0.99	50.15 ± 1.04	770.2 ± 67.0	70.8
S68-K	11.20 ± 1.65	30.28 ± 2.20	29.79 ± 5.66	26.10 ± 2.85	1624.4 ± 104.6	83.6
S68ipa-K	10.81 ± 0.16	54.84 ± 1.82	5.32 ± 0.28	26.75 ± 2.55	1584.1 ± 37.7	83.3
N-K	9.69 ± 0.81	17.35 ± 0.57	12.40 ± 0.98	62.08 ± 0.86	809.7 ± 56.3	69.4
Nipa-K	8.54 ± 0.93	18.16 ± 1.03	13.78 ± 1.20	60.86 ± 1.11	792.3 ± 30.8	73.3

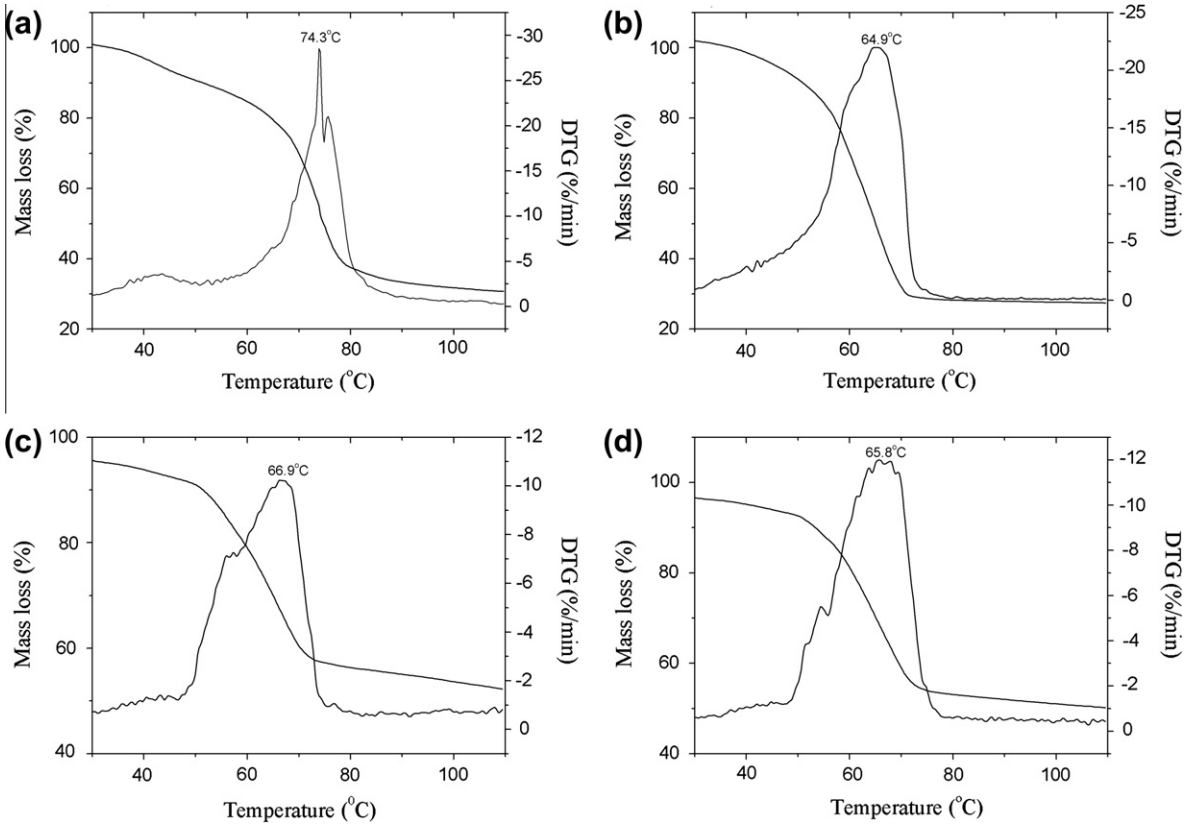

Fig. 4. TG and derivative TG profiles of test and reference emulsion systems: (a) S68, (b) S68ipa, (c) N and (d) Nipa.

Table 4
pH values and conductivity of placebo and active samples, assessed 7, 30 and 90 days upon preparation.

Sample	pH			Conductivity (μS/cm)		
	7 days	30 days	90 days	7 days	30 days	90 days
S68	4.71	4.33	4.25	13.86	8.44	5.32
S68ipa	4.79	4.47	4.54	7.53	4.35	3.46
N	4.30	3.82	3.68	5.19	4.98	3.20
Nipa	4.50	4.08	3.95	5.40	5.85	2.66
S68-K	4.40	4.19	4.15	20.43	13.08	15.64
S68ipa-K	4.30	4.25	4.36	17.92	10.16	14.47
N-K	4.13	3.95	3.85	3.64	3.13	3.60
Nipa-K	3.84	3.77	3.68	2.94	2.57	3.28

ture, *in vitro* permeation study was subsequently performed. However, the results correlated well with release profiles obtained by initial screening. Even less decisive IPA's permeation promoting effect was observed, since the profiles of basic and corresponding IPA-loaded samples were nearly superimposable when ASCs were used as the membrane (Fig. 5b). As expected, the amounts permeated through ASCs were nearly twofold higher than through excised human SC. However, the permeation coefficients for samples S68 and S68ipa were in good agreement: 7.91 ± 0.78 and 7.52 ± 0.18, 6.13 ± 1.72 and 8.24 ± 1.24 [10^{-6} cm s^{-1}], for ASCs and excised human SC, respectively. Although, in the long run, IPA addition led to somewhat enhanced ketoprofen permeation through isolated SC, it could not still be considered statistically significant when compared to the permeated amounts from sample S68-K (Fig. 5b) nor the reference samples (data not shown). This could be correlated with the obtained Cs values since the Cs of ketoprofen in APG-base with IPA ($Cs_{S68ipa} = 5.05 \times 10^{-4}$ g ml^{-1}) was threefold higher than in the corresponding base without alcohol ($Cs_{S68} = 1.66 \times 10^{-4}$ g ml^{-1}). As for reference samples, obtained saturation concentrations were lower (1.24 and 2.12×10^{-4} g ml^{-1}, for N and Nipa, respectively), indicating the marked influence of the APG colloidal structure on the drug's thermodynamic activity.

It seems reasonable to conclude that APG-based liquid crystalline structure acts as a potent diffusional barrier, thus controlling ketoprofen release. Although the influence of the two different colloidal structures on release characteristics was expected to be diverse, it could be stated that high viscosity, the prevailing characteristic of referent pharmacopoeial vehicle, induces ketoprofen release comparative to complex viscoelastic gel network that is formed in case of investigated APG-stabilized bases. These findings imply that the rate of drug delivery from APG-based emulsion systems is a complex parameter that cannot be correlated merely with the decrease in the viscosity.

The complexity of the very process of percutaneous absorption was further investigated *in vivo* through the tape stripping procedure in order to assess the influence of the colloidal structure of the test and reference bases (with and without the addition of IPA – 10% w/w) on the stratum corneum permeability. The rational for percutaneous absorption evaluation was twofold: first, as an *in vivo* assessment that depicts real in-use conditions with minimum discomfort and relative ease [44,45] and second, in order to evaluate ketoprofen delivery in the light of skin-vehicle interactions, especially regarding alcohol addition [46].

The extent of ketoprofen delivery into the stratum corneum was clearly and significantly increased from S68-K and S68ipa-K, relative to that achieved from N-K and Nipa-K (Fig. 6a). This is an obvious influence of the vehicle composition, i.e. the distinct colloidal structures of the assessed bases. However, this could not be easily correlated with the obtained Cs values. Due to the fact that the *in vivo* study was conducted in such a manner to mimic real in-

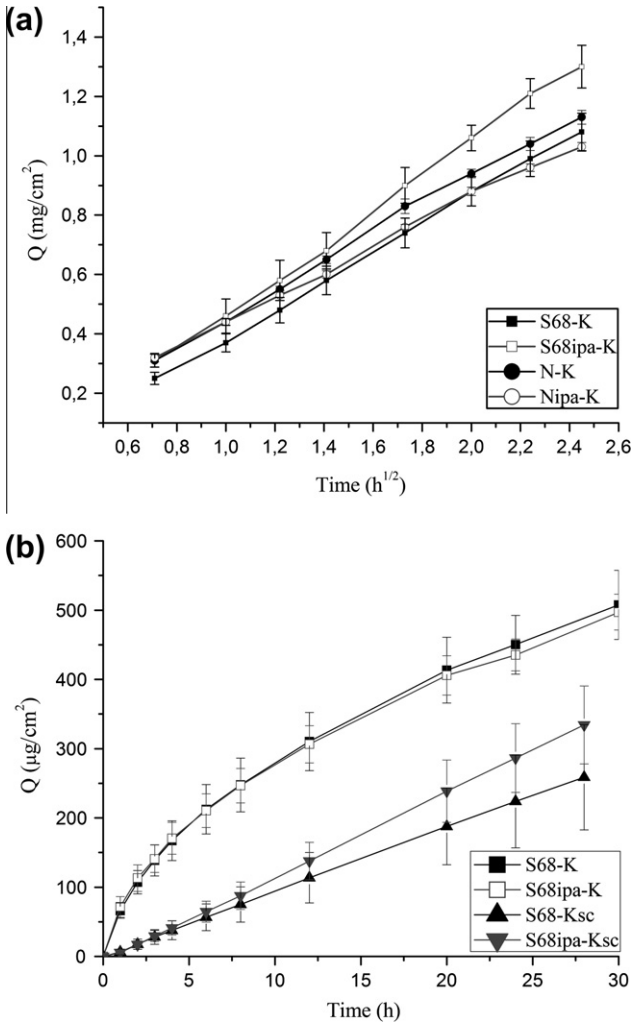

Fig. 5. *In vitro* biopharmaceutical characterization: (a) preliminary screening of ketoprofen release profiles following Higuchi equation (mean ± SD, $n = 3$); (b) cumulative amount of ketoprofen permeated through ASCs (labelled S68-K and S68ipa-K) and isolated SC (labelled S68-Ksc and S68ipa-Ksc) per unit surface area as a function of time (mean ± SD, $n = 6$ and 4, respectively).

use conditions (finite dosing, without occlusion), it could therefore be expected that some changes in composition have occurred and that super-saturation was inevitable.

It is generally accepted that an increase in the removed mass indicates a decrease in the cohesion of SC [47]. However, this clearly was not the case for IPA-loaded samples, since S68ipa led to smaller amounts of SC removed (obtained by Eq. (1)) in comparison with S68, especially by the first 3 adhesive strips (Table 5). After a 2-h penetration time, the amounts harvested from the 12th tape were $0.78 \pm 0.40\ \mu g/cm^2$ in case of S68-K and $0.83 \pm 0.28\ \mu g/cm^2$ for sample S68ipa-K. A relative enhancement ($p > 0.05$) in drug uptake from IPA containing samples correlates well with previously discussed *in vitro* findings.

Alongside tape stripping as a dermatopharmacokinetic procedure, TEWL was simultaneously employed as a technique that determines SC barrier function and indicates inter- and intraindividual variation in SC thickness. The average baseline (prior to tape stripping), middle (after 7th tape) and final (after 12th tape stripped) TEWL values are presented in Table 5. An average of 2.16-fold decrease in skin barrier function was recorded. Once again, the nature of the S68 and N bases' colloidal structure had a more pronounced influence on TEWL, rather than the addition of IPA.

Such cases where the difference obtained *in vivo* was statistically significant (Fig. 6) unlike data that stemmed from *in vitro* or even *ex vivo* studies have already been reported for ketoprofen as a model drug by Lodén et al. [12]. The easiest explanation lies in various absorption routes that are available *in vivo*. However, unlike Lodén, we cannot hypothesize that the difference is due to alcohol evaporation. Here, the difference in drug uptake is clearly related to the properties of emulsion base used. For that reason, a lower ketoprofen permeation observed through mean tape stripping profiles of N-K and Nipa-K could be correlated with their rheological properties (higher viscosity), and the fact that various factors, such as vehicle composition and its ultimate influence on skin hydration [48], can determine the drug uptake *in vivo*. Therefore, we were interested in evaluating the skin performance of investigated bases *in vivo*, alongside the dermal irritancy testing.

3.3. Skin irritation potential assessment – in vitro/in vivo safety considerations

During the characterization of a novel excipient or the development of new topical formulations, the investigation of dermal irritation potential is considered compulsory, being one of the most common adverse effects [30]. Skin irritation was assessed *in vitro*, using the MTT cytotoxicity assay as an established protocol for screening formulations prior to human *in vivo* dermatological evaluation. The rational for cytotoxicity testing was twofold: to evaluate the effect of suggested formulations stabilized with the novel cetearyl glucoside and cetearyl alcohol mixed emulsifier, on the one hand, and the addition of IPA, on the other. Biomarker used in the chosen *in vitro* irritation model is the reduction of the yellow tetrazolium salt (MTT) to a purple formazan dye by various dehydrogenase enzymes present in viable, metabolically active cells. Therefore, the presence of an irritant will result in a corresponding decrease in mitochondrial activity that will be detected by this colorimetric assay [49].

If taken that the cut-off limit defined for relative cell viability is 50%, both the basic APG formulation (S68) and IPA-loaded one (S68ipa) can be classified as non-irritant [30,31,50]. The adverse effects following the treatment with samples S68 and S68ipa were weak, i.e. no significant changes in cell viability could be observed (cell viabilities were in the range of 95.3% and 106.5%). Although it was expected that the increase in the concentration of the applied samples will induce the corresponding decrease in cell viability, no such tendency could be observed. Moreover, in both samples, a slightly lower viability was found after the application of 2.5% sample dispersion: 95.9% and 96.4%, for S68 and S68ipa, respectively.

Highly favorable effects of test samples on cellular viability *in vitro* are in accordance with previously published results of alkyl polyglucosides' mild nature [23]. However, comparative mildness of IPA containing sample was not anticipated and could only be attributed to the prevailing influence of the base itself, at least in the alcohol concentration used.

The cytotoxicity evaluation conducted on a three-dimensional human skin model is considered to mimic well the conditions which cells experience *in vivo* [51]. Nevertheless, the results are commonly discussed in correlation with those obtained by subsequent *in vivo* skin performance evaluation.

Investigated samples showed overall satisfying safety profiles. Three hours after occlusion removal, there was no significant change in EI, TEWL (Fig. 7a) or pH readings (Fig. 7b). As for SCH, all samples led to an increase in this parameter (Fig. 7b). Admittedly, the increase could only be considered significant in case of N and Nipa samples, probably due to the vehicles' highly occlusive effect. It should be pointed out that for S68 vehicle there was a lack of TEWL increase, while a trend of increase was present for S68ipa,

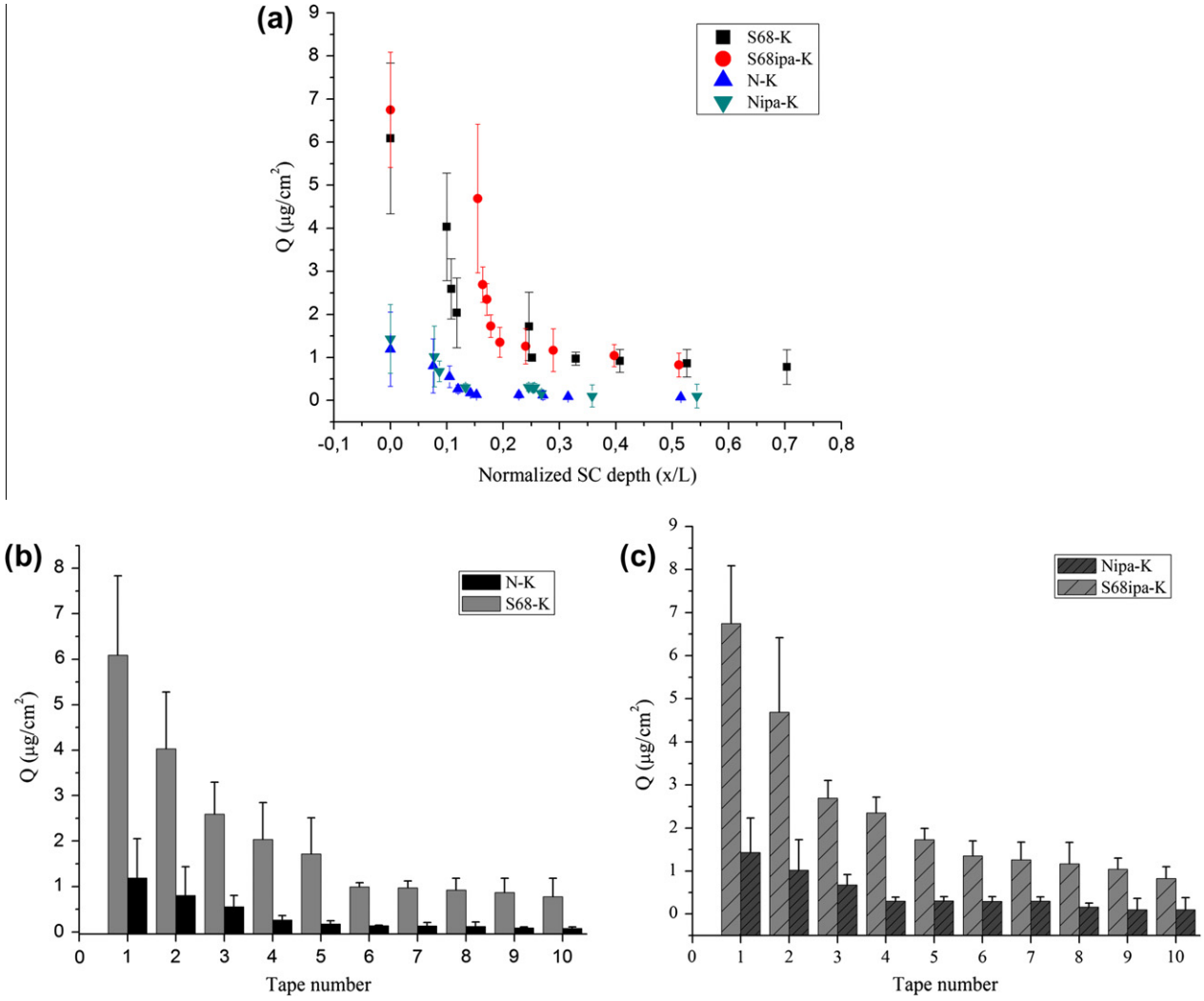

Fig. 6. Penetration profiles of ketoprofen emphasizing the influence of the colloidal nature of the base on *in vivo* skin absorption: (a) comparative ketoprofen penetration profiles assessed through tape stripping technique across the SC path-length L (mean ± SD, $n = 6$); Concentration of ketoprofen (μg/cm^2) in different skin layers – influence of the applied base: (b) S68-K vs. N-K and (c) S68ipa-K vs. Nipa-K. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 5
Parameters assessed through tape stripping procedure: the amount of drug retained in different skin layers (μg cm^{-2}), removed protein fraction (μg) and TEWL as a parameter of skin barrier integrity (g m^{-2} h^{-1}), all expressed as mean ± SD.

Sample	Q (μg cm^{-2})			T (μm)			TEWL (g m^{-2} h^{-1})		
	Q_{3-5}	Q_{6-8}	Q_{9-12}	T_{3-5}	T_{6-8}	T_{9-12}	$TEWL_0$	$TEWL_7$	$TEWL_{12}$
S68-K	12.71 ± 1.23	4.75 ± 0.56	3.54 ± 0.28	9.73 ± 1.87	3.84 ± 1.07	3.01 ± 0.67	6.48 ± 2.94	10.85 ± 2.55	12.25 ± 1.34
S68ipa-K	13.88 ± 1.16	5.42 ± 0.33	4.29 ± 0.36	5.15 ± 0.29	2.56 ± 0.28	2.91 ± 0.37	5.12 ± 1.98	9.40 ± 1.19	12.08 ± 1.06
N-K	2.55 ± 0.30	0.58 ± 0.41	0.42 ± 0.13	4.84 ± 1.23	2.20 ± 1.63	1.72 ± 0.80	3.87 ± 0.65	7.65 ± 2.00	8.58 ± 3.50
Nipa-K	3.13 ± 0.12	0.91 ± 0.56	0.67 ± 0.17	5.35 ± 0.71	3.45 ± 0.72	2.56 ± 0.47	4.12 ± 1.09	8.92 ± 3.59	8.99 ± 2.50

N and Nipa. This implies enhanced compatibility of the APG basic vehicle with the skin barrier.

4. Conclusions

Topical drug preparations based on surfactants of natural origin are in high demand for some time. The group of APG emulsifiers has especially been considered prospective in both pharmaceutical industry and compounding practice and is being recognized by regulatory agencies. The conducted characterization of cetearyl glucoside and cetearyl alcohol stabilized bases proved them to be promising ready-to-use vehicles in pharmaceutical compounding.

APG-based emulsion systems (prospective pharmaceutical bases) showed highly satisfactory ability to remain physically stable upon the addition of isopropyl alcohol, without notable impact on overall stability of the emulsion system. This could be of great importance in *ex tempore* preparation of various topicals, particularly with poorly soluble drugs. In contrast to *in vitro* (nearly superimposable release profiles), *in vivo* measurements show superior ketoprofen skin absorption from APG bases related to corresponding reference samples (N bases), irrespective of the anticipated IPA

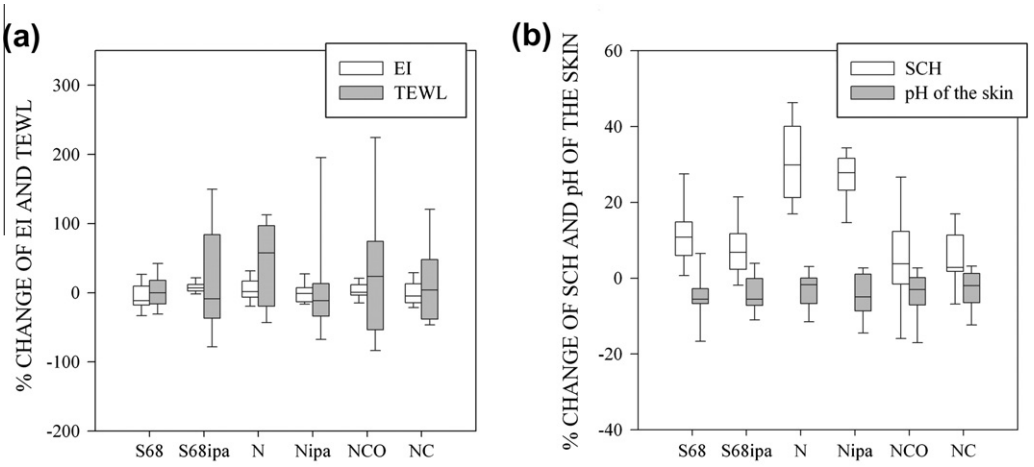

Fig. 7. *In vitro*/*in vivo* safety assessment of placebo samples: percentage change in (a) EI and TEWL, (b) SCH and pH of the skin, after occlusion removal vs. baseline measurements, for investigated samples, non-treated control under (NCO) and without occlusion (NC).

influence to the drug penetration-permeation profile. Overall, satisfactory biopharmaceutical properties and safety *in vitro*/*in vivo* tested profiles were obtained for APG-containing prospective pharmaceutical bases when compared to traditionally used Non-ionic hydrophilic cream, a pharmacopoeial base of proven quality. Taking also into account the APG bases' distinctively ameliorated sensory characteristics (confirmed by rheological assessment), a fact that often prevails in aspect of patient compliance, it could be concluded that the investigated APG bases could be considered as a preferential option in drug compounding related to the conventional ones.

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