https://doi.org/10.31925/farmacia.2026.1.17 **ORIGINAL ARTICLE**

SPIRONOLACTONE QUANTITATIVE DETERMINATION AND *IN VITRO* RELEASE STUDY FROM TOPICAL EMULSIONS

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Abstract

Topicals are often the first choice for treating skin diseases. Dermal absorption tests are applied to predict the ability of an active to permeate through the skin. Spironolactone has been used as a valuable *off-label* treatment option in both systemic and topical acne therapy. Alkyl polyglucoside (APG) based emulsions with spironolactone showed acceptable skin irritation profiles. The main goals of this study were to examine the content and release profile of spironolactone from APG-based emulsions. Four different APG-based emulsions were prepared as carriers for topical spironolactone; two contained glycolic acid in addition to spironolactone, to test its potential action as a penetration enhancer. The spironolactone content was determined by the HPLC method. An *in vitro* release study was performed using vertical Franz diffusion cells. Results showed that spironolactone release from the emulsions and permeation through the polycarbonate membrane depended on the APG emulsifier, as well as on the composition of the emulsion itself. It is also assumed that emulsions stabilized with arachidyl glucoside and arachidyl behenyl alcohol as emulsifiers can lead to extended release of incorporated spironolactone.

Rezumat

Spironolactona este un agent terapeutic utilizat off-label atât în terapia sistemică, cât și în cea topică a acneei. Emulsiile bazate pe alchil-poliglucozide (APG) formulate cu spironolactonă au demonstrat un profil acceptabil de iritație cutanată. Obiectivele principale ale acestui studiu au fost evaluarea conținutului și a profilului de eliberare a spironolactonei din emulsii pe bază de APG. Au fost formulate patru tipuri distincte de emulsii APG utilizate ca vehicule pentru administrarea topică a spironolactonei; două dintre acestea au inclus, suplimentar, acid glicolic, pentru investigarea potențialului său rol de promotor al penetrării cutanate. Conținutul de spironolactonă a fost determinat prin cromatografie lichidă de înaltă performanță (HPLC). Studiul de eliberare *in vitro* a fost realizat utilizând celule de difuzie Franz verticale. Rezultatele au indicat că eliberarea spironolactonei din emulsii și permearea prin membrana de policarbonat sunt influențate atât de tipul emulgatorului APG, cât și de compoziția sistemului emulsionat. Se presupune, de asemenea, că emulsiile stabilizate cu arachidyl glucoside și arachidyl behenyl alcohol ca emulgatori pot favoriza o eliberare prelungită a spironolactonei încorporate.

Keywords: spironolactone, alkyl polyglucoside emulsifiers, *in vitro* release study, off-label acne treatment

Introduction

Acne vulgaris is a common chronic inflammatory skin disease that affects sebaceous glands on the face, upper back, upper chest, and shoulders (1, 2). Due to clinical manifestations, it is often associated with psychological disturbances and depression (3, 4). The pathogenesis of acne involves androgen hormones, which play an important role in the follicular hyperkeratinisation of sebaceous glands, the increased sebum production, and proliferation of *Cutibacterium acnes* (*C. acnes*) with associated inflammation (5). Sebaceous glands are rich in androgen receptors (6-9). Some steroid and non-steroid drugs have affinities for androgen receptors (10). Dihydrotestosterone (DHT), formed by the 5 α-reduction of testosterone in the sebaceous glands, is the most potent androgen (11, 12). Acne therapy can be both oral and topical; the most commonly used topical therapies are antibiotics (clindamycin, erythromycin) and retinoids (tazarotene, tretinoin, adapalene), while the most commonly used oral drugs are antibiotics (macrolides, tetracyclines), oral contraceptives, isotretinoin, and androgen receptor blockers in women (13). Additionally, dermocosmetics and chemical peeling with alpha-hydroxy acids, such as glycolic acid (GA), have been shown to be effective in treating acne (14). Besides that, there is evidence of the positive effect of GA as a penetration enhancer. GA showed a positive effect on the penetration of active substances with log $P < 2.89$ by increasing skin hydration, which led to an increase

in partition into the skin of hydrophilic or low lipophilic compounds (15).

Spironolactone (SP) is a steroidal, potassium-sparing diuretic. As an antiandrogen, it can block DHT receptors and reduce sebum secretion, the key factors in acne, so it has been used as systemic *off-label* therapy. In order to avoid numerous side effects of systemic administration, topical SP was proposed as an effective therapeutic option in acne (16-18).

The skin is an effective barrier between the human body and the external environment. It regulates homeostasis and body temperature, particularly by limiting transepidermal water loss (TEWL) (19-22). Topical preparations are the first choice in the local treatment of skin diseases, having in mind low systemic exposure and good patient compliance (23). However, special attention should be paid to the choice of a suitable vehicle.

Dermal absorption tests are routinely applied to predict the ability of an active therapeutic ingredient to permeate through the skin to the site of action. Therefore, there is a growing demand for reproducible and reliable *in vitro* and *ex vivo* skin absorption tests. The Organization for Economic Co-operation and Development (OECD) and the United States Environmental Protection Agency developed guidelines for *in vivo* and *in vitro* assessment of percutaneous absorption of drugs (24).

High Performance Liquid Chromatography (HPLC) can be used to determine the drug concentration in a preparation or product. In the implementation of this method, optimization must be performed using a column and a mobile phase capable of separating the mixture in order to establish a sensitive, precise, selective, and robust HPLC method that can produce a fast and efficient separation (25). There are available data about HPLC analysis of SP from different pharmaceutical forms (26-29), but topical semi-solids with SP were not analyzed. In this regard, we aimed to develop an HPLC method for the SP determination from topical emulsions, to examine its content in the final preparation.

Local skin disease therapy provides a high level of drug at the site of action (30, 31). Alkyl polyglucoside (APG) emulsifiers are a group of natural, nonionic, polyethylene glycol (PEG)-free emulsifiers. Our previous study confirmed the safety of topical application of 5% SP preparations based on different APG emulsifiers (32). In addition to the absence of reported methods of SP content analysis, there are no data on methods predicting the *in vitro* SP release from the topical emulsion either. As a continuation of our previous research (32, 33), the main goals of the current study were to examine SP content in the emulsion, as well as the SP release profile from the emulsions and the potential influence of GA. In that aim, we also had to establish a sensitive, robust, selective, and precise HPLC method for the estimation of SP content in emulsions, as well as to develop a method that evaluates *in vitro* SP release from the emulsion using Franz-diffusion cells.

Materials and Methods

Reagents

SP was provided as a donation by "Galenika" (Zemun, Serbia), HPLC-grade methanol was purchased from Sigma-Aldrich (St. Louis, MO, USA). Cetearyl glucoside and cetearyl alcohol as well as Arachidyl glucoside and Arachidyl glucoside and arachidyl behenyl alcohol were donated by "Seppic" (Paris, France), while cetostearyl alcohol, caprylic/capric triglycerides, isopropyl myristate, cyclomethicone, glycerol, ethanol 96%, glycolic acid, and preservative were purchased from Zorkapharma (Sabac, Serbia).

Preparation of SP emulsions

According to the results of our study (32), we prepared four different samples of oil-in-water (O/W) emulsions with different APG emulsifiers as carriers for SP (Table I).

Table I

Ingredients of the tested samples

Ingredient	F1 (%m/m)	F2 (%m/m)	F3 (%m/m)	F4 (%m/m)
Arachidyl glucoside and arachidyl behenyl alcohol		10		10
Cetearyl glucoside and cetearyl alcohol	7		7	
Caprylic/Capric Triglycerides	10	10	10	10
Cetostearyl alcohol	2	2	2	2
Cyclomethicone		10		10
Isopropyl myristate	10		10	
Spironolactone	5	5	5	5
Glycerol	2	2	2	2
Glycolic acid			4	4
Ethanol 96%	5	5	5	5
Preservative	0.5	0.5	0.5	0.5
Purified water	ad 100	ad 100	ad 100	ad 100

Cetearyl glucoside and cetearyl alcohol, certified by the Food and Drug Administration (FDA) as an excipient for pharmaceutical use (34), and arachidyl glucoside and arachidyl behenyl alcohol, especially

recommended for oily skin care preparations (35), have been used. Samples F1 and F2 contained only 5% of SP dispersed in the vehicle, while samples F3 and F4 also had GA, to test its ability to act as a penetration enhancer (Table I). Samples were prepared by heating and stirring in a laboratory glass. For samples containing cetearyl glucoside and cetearyl alcohol as an emulsifier, the oily phase was heated to 70°C (magnetic stirrer (IKAMAG IKA, Germany)), after which it was added under stirring (800 rpm) (propeller laboratory stirrer (RV16 basic IKAVR VERKE, Germany)) to the aqueous phase at a temperature of 72 - 75°C. Stirring at a constant temperature was continued for the next 5 minutes at 800 rpm, followed by another 3 minutes at 500 rpm. After the emulsification was completed, cooling was started under stirring at 300 rpm, when a preservative and ethanol 96% were added (under 40°C). Stirring was maintained during the gradual and controlled cooling of the emulsions until complete cooling, without affecting lamellar phase formation. Samples containing arachidyl glucoside and arachidyl behenyl alcohol as emulsifier were prepared in the same way, with the heating of the oily phase to 80°C, according to the recommendation of the emulsifier manufacturer. The aqueous phase was heated to 82 - 85°C. The emulsions containing GA were neutralized by the controlled addition of triethanolamine with continuous pH monitoring using a pH meter (HI 8417, Hanna Instruments, Woonsocket, RI), to achieve a final emulsion pH in the range of 3.7 - 3.8. This pH range has been demonstrated to be the most suitable for the topical application of formulations containing glycolic acid (36, 37). At the end, 5% of SP was dispersed in an appropriate amount of emulsion for all tested samples. Each formulation was prepared once, as the formulation parameters were previously optimized and validated in our earlier study, where the reproducibility of the emulsification process and the resulting physicochemical properties were systematically confirmed (32, 33). Based on those findings, the same experimentally established conditions were applied in the present study, ensuring consistent and reproducible emulsion characteristics.

Preparing standard solutions and sample solutions

SP stock standard solution was prepared by dissolving the accurately weighed amount of standard substance (Ph. Eur. 10) in methanol to give a final concentration of 0.4 mg/mL. The working standard solution was made by diluting the required quantity of stock solution with methanol to achieve the SP concentration of 5 µg/mL. The standard solution was analyzed by HPLC (38).

An equal mass of all emulsions was measured, dissolved in 10 mL of methanol, and stirred for 10 minutes using a vortex (38). After centrifugation at room temperature, the supernatants were subjected to solid phase extraction (SPE) in order to provide more effective separation of SP from interfering compounds. SPE was carried out on C18ec cartridges (CHROMABOND® RP development kit II, Macherey-Nagel, GmbH, Düren, Germany) by using a vacuum manifold (Visiprep™ SPE Supelco 57030-U, Sigma-Aldrich Chemie GmbH, Eschenstrasse, Germany). Before extraction, cartridges were conditioned and equilibrated with 1 mL of methanol and 2 mL of deionized water. After that, one milliliter of the prepared supernatant was loaded on the cartridge at a flow rate of 1 mL/min. The SP elution was achieved in two cycles, with 1 mL of methanol each. Final analyte solutions were diluted with methanol to achieve the expected concentration corresponding to the concentration of the standard solution (5 µg/mL) and then analyzed by HPLC.

HPLC conditions

For HPLC analysis, an Agilent Technologies 1200 Series Apparatus (Santa Clara, CA 95051, USA) with a diode array detector was used, while Agilent ChemStation software was used for chromatogram monitoring and analysis. Chromatographic separation was performed on a Restek Ultra IBD C18 analytical column (150 × 3 mm, 3 µm). The column working temperature was 35°C. A mixture of methanol (A) and deionized water (B) was used as the mobile phase, with the volume ratio of 60:40, respectively. The pH of the mobile phase was experimentally determined using a calibrated pH meter and was found to be 6.5. The mobile phase was degassed by means of the built-in online vacuum degassing system of the Agilent 1200 Series apparatus before chromatographic analysis. The injection volume of the sample was 10 µL, while the flow rate of the mobile phase was set at 0.45 mL/min. The wavelength used of UV detector was 238 nm. SP was identified by chromatographic comparison with the reference standard.

In vitro release study

Vertical Franz diffusion cells (n = 6; Gauer Glas, D-Püttlingen, Germany) were used to perform an *in vitro* release study to determine SP release kinetics from these semisolids. Considering low SP solubility, after detailed analysis and its stability evaluation in different potential acceptor phases, ethanol 50% was chosen, since it could prevent degradation and ensure adequate solubilization. According to established *in vitro* release testing methodologies using Franz diffusion cells, cumulative drug release of ≤ 30% of the applied dose over the test duration has been demonstrated to satisfy the requirements of a standard *in vitro* release testing (39). The suitability of 50% ethanol as the receptor medium was confirmed by evaluating the solubility and stability of SP under experimental conditions. The drug concentration in the receptor medium was determined using an HPLC method immediately after dissolution, and after 24 h and

48 h. The results demonstrated that SP remained stable and fully dissolved throughout this period, with concentrations substantially exceeding the requirements sufficient for *in vitro* release studies. These findings validated 50% ethanol as an appropriate receptor medium and confirmed the maintenance of sink conditions during Franz diffusion experiments. Cell receptor chambers were filled with ethanol 50%, heated to 32˚C (chamber volume 12 mL; effective diffusion area 2.01 cm^2). We used polycarbonate membranes (Nuclepore™, Whatman, Maidstone, United Kingdom; pore diameter 0.1 µm, thickness 25 µm, surface area 2.01 cm^2), activated in the same ethanol solution during 12-hours period. They were carefully placed and fastened between the donor and acceptor chamber, and any existing air gaps were removed. Membrane integrity was confirmed by visual inspection before use, while pore size uniformity and membrane thickness were ensured according to the manufacturer's technical specifications. The donor chamber was filled with 1 g of the samples being investigated, and subsequently covered with silicone film (Parafilm™, USA) to prevent evaporation and loss of the formulation during sampling. All formulations were prepared with identical nominal SP content, ensuring identical theoretical drug doses in permeation experiments. Cells were placed in the water bath where the temperature of 32˚C was maintained throughout the experiment, and were continuously stirred using a magnetic stirrer at 500 rpm. The release study was conducted over 6 hours. Aliquots of 0.6 mL of the acceptor phase were withdrawn at 6 predetermined time points (0.5 h, 1 h, 2 h, 3 h, 4 h, and 6 h), and immediately replaced with the same volume of fresh and thermostated ethanol solution in order to maintain constant volume and sink conditions. The HPLC technique was used to determine the SP content in all samples. Given the identical applied doses, permeation data were primarily evaluated relative to the theoretical SP content to enable direct comparison between formulations. In order to find the best model that describes the drug release profile from tested emulsions, the cumulative amounts of SP *per* unit area were evaluated through several kinetic models.

Statistical analysis

All experiments were performed in six replicates (n = 6), and the results are presented as mean ± standard deviation (SD). Statistical analysis was performed using IBM SPSS Statistics (version 23, IBM Corp., Armonk, NY, USA). The effects of formulation and time on cumulative permeation were evaluated using two-way analysis of variance (two-way ANOVA). When statistically significant differences were detected, post hoc multiple comparisons were performed using Tukey's test. A p-value < 0.05 was considered statistically significant.

Results and Discussion

Physicochemical characteristics of the investigated emulsions

The investigated emulsions were prepared using identical methodology and composition principles as previously described (32). Although detailed droplet size distribution and rheological characterization were not within the scope of the present permeation-focused study, all formulations were macroscopically homogeneous and physically stable, with no evidence of phase separation during the experimental period. The pH and electrical conductivity values of the tested emulsions remained within the range recommended for products intended for skin application, as well as characteristic of emulsions with lamellar liquid crystals (32). Also, polarization micrographs of all samples showed the presence of deformed Maltese crosses, indicating the presence of a liquid-crystalline phase, even after SP dispersion (32). Taken together, the pH, conductivity, microscopic, and stability data provide adequate evidence of structural comparability among the investigated formulations for the purposes of this study.

Considering the standardized preparation procedure and comparable ingredients of the emulsions, the systems are considered structurally comparable for the purpose of assessing relative permeation.

Chromatographic separation and optimization of the HPLC conditions

An analytical C18 column was chosen due to the SP lipophilicity. In the initial development of the method, several preliminary trials were conducted with different volume ratios of methanol (solvent A) and deionized water (solvent B) to optimize the retention time, resolution, peak shape, and other chromatographic parameters. Thus, the volume ratio of solvent A to solvent B varied from 30:70 to 65:35. The operating column temperature was set between 25 and 35°C. The elution was performed at a flow rate of 0.4 - 0.9 mL/min, and the injection volume was 5 - 20 µL. The wavelength of the UV detector was set at 238 nm, according to Ph. Eur. 9. A mobile phase consisting of methanol and deionized water in the ratio of 60:40 (%v/v) was found to be optimal. The optimal working temperature and the flow rate were 35°C and 0.45 mL/min, respectively. The optimal injected sample volume was 10 µL to achieve the required limit of quantification (LOQ). Under the selected conditions, SP was fully separated and determined with a good peak shape after around 4.5 minutes. Representative chromatograms of the samples are shown in Figure 1.

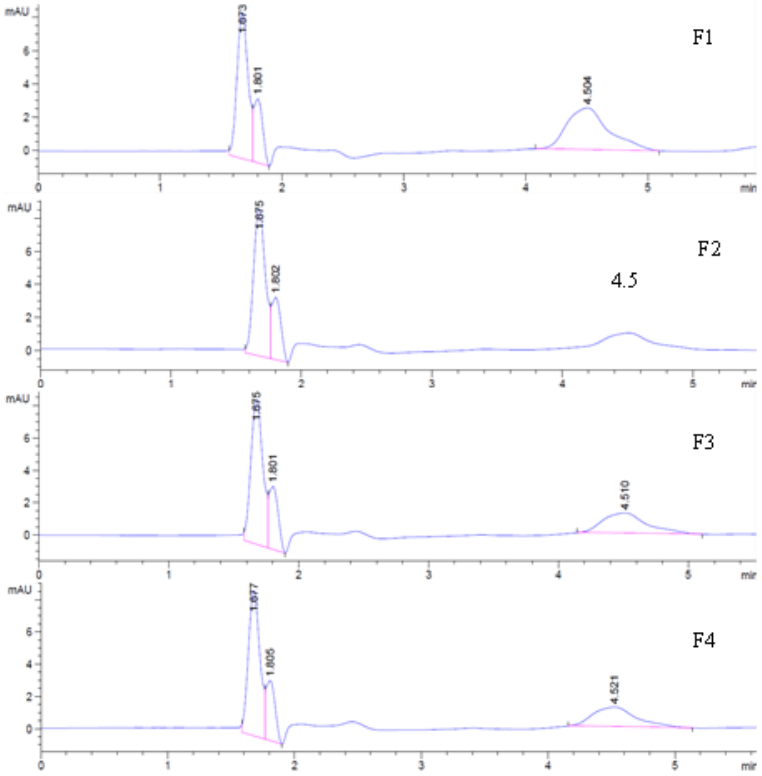

Figure 1.
Chromatograms of the investigated samples

Very selective separation was achieved for SP determination in all samples. No interferences were observed in the region around SP, and high purity of the UV spectrum of SP peaks from samples was obtained, too.

Validation of the HPLC and SPE method

Parameters of the analytical method for SP determination, such as selectivity, linearity, the limit of detection (LOD), the limit of quantification (LOQ), accuracy, and precision, were validated. The method validation was conducted according to ICH guidelines (International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH). ICH guideline M10: Bioanalytical method validation and study sample analysis 2022 (40). The selectivity of the method was evaluated by analyzing the chromatograms of placebo samples and those of prepared emulsions. Placebo samples and SP emulsions were previously subjected to solid-phase extraction. The potential interferences from the emulsion matrix were estimated by analysing the SP peak resolution and peak purity. The peak purity was calculated by Agilent ChemStation software for the UV spectrum of SP peak obtained by the DAD detector. In the chromatograms of the placebo mixture, no peak appears at the retention time of SP. Therefore, the mixture of excipients used in the compounded emulsions did not affect the chromatographic analyses of SP. There is no interference from excipients, and the method is of appropriate selectivity. Also, a high-purity peak of SP was obtained by analyzing the peak UV spectra in the chromatogram of the emulsion.

The linearity of the method was evaluated by constructing the calibration curve for five standard SP solutions in the concentration range of 1.0 - 9.0 µg/mL. The average peak area of three replicate injections at each concentration against SP concentration was used for each data point. Linear regression analysis was conducted to calculate the intercept, slope, and linear regression coefficient (R^2) using OriginPro 6.1. Software (OriginLab Northampton, Massachusetts, USA). The following equations were used to calculate the limit of detection and the limit of quantification: LOD = 3.3 Sd/b and LOQ = 10 Sd/b, where Sd is the standard error of the intercept of the regression line, and b is the slope of the calibration line. Analytical parameters of the HPLC method are presented in Table II.

Table II
Analytical parameters of the HPLC method

Parameters	
Number of points	5
Linearity range (µg/mL)	1.0 - 9.0
Slope ± SD	60.51 ± 1.72
Intercept ± SD	15.11 ± 9.85
Linear regression coefficient (R^2)	0.997
LOD (µg/mL)	0.54
LOQ (µg/mL)	1.63

SD - Standard deviation

The value of the correlation coefficient, higher than 0.99, indicates good linearity of the calibration graph.

The examination of the system suitability parameters was conducted in terms of retention time, peak area, the number of theoretical plates, capacity factor (k′), peak symmetry, resolution, and selectivity. The number of theoretical plates, peak symmetry, capacity factor, and resolution were obtained from chromatograms by using Agilent ChemStation software. Intra-day precision of retention times and peak areas was calculated on a single day using three replicates of the one sample and was expressed as relative standard deviation (RSD, %). The chromatographic parameters, as well as repeatability of the retention time and peak areas for standard and F1 sample solutions, are summarized in Table III.

Table III

HPLC system suitability parameters for C18 column

	Rt ± SD (min)	**Mean area ± SD**	**Number of theoretical plates**	**Peak symmetry**	**k'**	**Resolution**	**Selectivity**
Standard	4.49 ± 0.013 (RSD 0.3%)	316.2 ± 9.13 (RSD 2.9%)	1210	0.83	2.17	7.31	8.07
Sample (F1)	4.51 ± 0.0049 (RSD 0.1%)	44.6 ± 7.2 (RSD 16.1%)	1017	0.87	2.20	7.67	7.99

The accuracy of the method was determined by extraction recovery studies. As SP is a lipophilic (fat-soluble) compound, a traditional cartridge of C18ec sorbents was used for SPE from emulsions. C18ec cartridge contains endcapped octadecyl phase and shows strong interaction with nonpolar, hydrophobic organic compounds. Also, Varin *et al*. used C18 sorbent for the extraction of SP from plasma samples (41).

The recovery of the SPE procedure was evaluated by applying the SPE method to a standard SP solution concentration of 5.0 µg/mL and a placebo emulsion. Recovery was calculated as the ratio of the found and nominal concentration of SP. The recovery of the SPE procedure for standard solutions of SP was found to be 84.5 ± 1.7% for a concentration level of 5.0 µg/mL. The placebo sample was treated with the same SPE procedure as the active sample, before the chromatogram was recorded. The chromatogram of the placebo sample did not show a peak at the retention time of SP, indicating no interference from excipients during extraction and chromatogram recording (Figure 2).

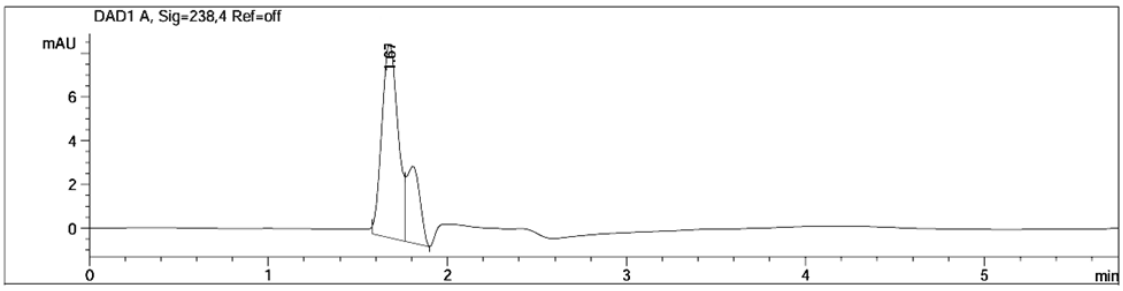

Figure 2.

Chromatogram of the placebo sample

It should be noted that SPE recovery was evaluated using standard SP solutions subjected to the extraction procedure, while spiked emulsions were not assessed. Consequently, potential matrix-related extraction inefficiencies cannot be fully excluded, and the absolute SP content in the formulations may therefore be underestimated. This represents a limitation of the present study.

HPLC analysis of APG emulsions with 5% SP

It was previously established that the formulation of specific lamellar liquid-crystalline and gel phases, structures that are able to incorporate large amounts of water, is responsible for the APG-based emulsions' stability (34, 42-43). Emulsions based on cetearyl glucoside and cetearyl alcohol and arachidyl glucoside and arachidyl behenyl alcohol with 5% SP showed good physical stability during a 30-day study and constant pH values, which are suitable for skin application (32). Also, APG-based emulsions with 5% SP showed acceptable skin irritation profiles, which could indicate safety during their topical application (32). These emulsions also showed good skin hydration potential, which is also very significant in acne therapy (32). In our study, the SP concentration obtained in all samples was lower compared to the nominal SP concentration (5%) (Table IV).

Table IV

Results of the HPLC analysis

	Retention time (min)	Nominal SP concentration in emulsions (mg/g)	Found SP concentration in analysed emulsions (mg/g)	Relative standard deviation (%)
F1	4.5 ± 0.05	50	9.5 ± 0.33	3.59
F2	4.51 ± 0.11	50	3.8 ± 0.27	7.65
F3	4.51 ± 0.08	50	4.6 ± 0.19	4.03
F4	4.52 ± 0.14	50	4.5 ± 0.37	7.86

The quantified values therefore, represent the extractable fraction of SP under the applied analytical conditions. No additional peaks corresponding to degradation products were detected in the HPLC chromatograms, and the retention time and UV spectral characteristics of SP remained consistent across all samples, indicating chemical stability of the active substance within the investigated emulsions. The reduced quantified content may be associated with the limited extractability of a fraction of SP from the structured emulsion matrix. Namely, it was previously shown (44-46) that a pronounced increase in the evaporation rate of APG-based emulsions (including the emulsions stabilized with both cetearyl glucoside and cetearyl alcohol, or arachidyl glucoside and arachidyl behenyl alcohol of similar composition of oil phase) between 70°C and 100°C indicated that a great amount of water was entrapped, either within the hydrophilic gel phase or as water fixed between lamellae of the liquid crystalline phase. Using different actives (both lipophilic and hydrophilic), the incorporation of different portions into the interlamellar spaces of the generated lamellar liquid crystalline structures placed around the dispersed oil droplets was shown, whereas the rest of the active was dissolved in the bulk water. So, we presumed that SP was also partly "entrapped" in the structure. Detailed structural investigations and comprehensive mass balance studies are planned to fully clarify the localization and extractability of SP within these systems. The reduced quantified SP content observed in the investigated formulations should be interpreted cautiously. The proposed explanation involving drug entrapment within the organized emulsion structure and consequent limited extractability represents a hypothesis rather than an experimentally confirmed mechanism. Given the lipophilic nature of SP and the structured character of the investigated systems, partial retention of the drug within the internal phase cannot be excluded. Although the analytical method showed satisfactory validation parameters, including acceptable recovery, incomplete extraction from complex colloidal systems remains a possible limitation. Therefore, the low quantified content is acknowledged as a limitation of the present study and may reflect restricted extractability rather than actual drug loss.

In fact, our previous study, which employed polarization microscopy as a method important for structural characterization (32), already showed the presence of deformed Maltese crosses, indicating the presence of a liquid-crystalline phase in samples investigated in this study, even after SP dispersion. However, that was not the objective of this paper, and detailed structural investigations and comprehensive mass balance studies will be conducted to fully clarify the localization and extractability of SP within these systems. Sample F2 showed lower quantified SP values compared to F1, suggesting possible differences in drug distribution due to the applied emulsifier. Also, the sample F2 showed a lower concentration of "free" SP compared to sample F1, which confirms that a larger amount of SP remained trapped inside the complex structure of lamellar liquid crystals of arachidyl glucoside and arachidyl behenyl alcohol as emulsifier, which contributes to an extended release of incorporated SP. The addition of GA to the emulsions and the subsequent regulation of the pH value (around 3.75) lead to larger SP peak areas in the HPLC analysis, indicating that formulation variables may affect SP extractability and distribution within the vehicle. Emulsions with arachidyl glucoside and arachidyl behenyl alcohol as emulsifiers confirmed this assumption, indicating that the addition of GA can lead to an improved release of SP from the vehicle. On the other hand, the results of HPLC analysis show that the addition of GA to emulsions with cetearyl glucoside and cetearyl alcohol leads to smaller mean areas, which may indicate a weaker release of SP (Table IV). Possible reasons for this outcome could be potential major changes in emulsion stability, as well as a more evident change in the partitioning behavior of SP after the addition of GA, compared to the emulsions stabilized with arachidyl glucoside and arachidyl behenyl alcohol as emulsifier. Nevertheless, further structural characterization is planned to confirm the underlying mechanisms.

In vitro release study

In the last two decades, it was emphasized that animal experiments should be avoided whenever scientifically feasible. *In vitro* screening of SP release was conducted as a technique commonly used in bioavailability evaluation of preparations intended for the topical application, as well as to find

the mechanism of active ingredient release and to determine the potential of a particular vehicle. This process is basically divided into three steps: 1) penetration, which consists of the entry of the chemical compound into the *stratum corneum*; 2) permeation, the gradual passage of the substance through the subsequent layers, both anatomically and functionally different from the *stratum corneum*; 3) the uptake into blood and lymphatic vessels (20, 47-51). Having in mind the low water solubility of SP, it was of interest to examine if the developed APG emulsion was able to improve its solubility, leading to an increase in dermal availability, as a prerequisite for therapeutic efficiency. In addition, this experiment served to investigate whether the preparation procedure of the tested emulsions (taking into account the differences between the used emulsifiers and the composition of the emulsions themselves), as well as the addition of GA, were discriminative in the extent and rate of SP release. The results are presented in Figure 3.

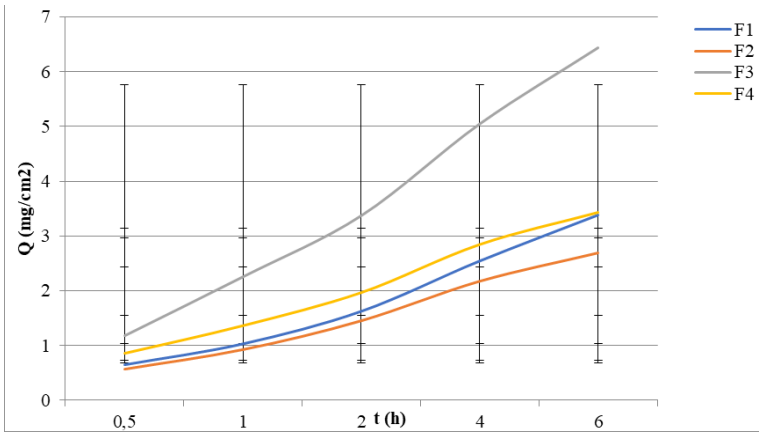

Figure 3.

Results of an *in vitro* screening of SP release profiles from investigated samples; Q – released amount of SP, t – elapsed time

The polycarbonate (0.1 µm) membrane was chosen as an inert barrier in accordance with standard *in vitro* release testing protocols, where it is widely recognized that such membranes do not significantly limit the diffusion of lipophilic drugs but rather serve to separate the donor formulation from the receptor medium (52). The pore size of 0.1 µm is much larger than the molecular dimensions of SP, making steric hindrance unlikely. All formulations were prepared with an identical nominal SP concentration (5% m/m) under the same manufacturing conditions. The cumulative permeation values are presented as absolute amounts permeated *per* unit area (mg/cm²). The cumulative amount of SP released after 6 h of the experiment ranged from 2.69 mg/cm^2 in F2 to 6.43 mg/cm^2 in F3. All data are presented as mean ± SD (n = 6). Although differences were observed in experimentally quantified SP content, all formulations were prepared with identical nominal drug loading. The extraction method demonstrated consistent recovery, suggesting that variability between formulations may reflect matrix-dependent extraction behavior rather than true differences in incorporated dose. It should be emphasized that the results interpretation was based on the nominal SP loading of the formulations. Although potential differences between nominal and experimentally quantified content may influence absolute release values, all formulations were prepared and evaluated under identical conditions. Therefore, comparative analysis of release performance among formulations remains reliable. Two-way ANOVA revealed a statistically significant effect of formulation ($p < 0.0001$), time ($p < 0.0001$), and their interaction ($p < 0.0001$), indicating distinct permeation profiles among the tested formulations. Post hoc Tukey's test confirmed that the cumulative amount of SP released from F3 was significantly higher compared to F2 at 6 h ($p < 0.001$). Since identical amounts of emulsions were applied in Franz diffusion experiments, permeation data were interpreted relative to the theoretical drug load. Importantly, comparative permeation profiles and kinetic interpretation were not affected by this approach. Based on the different lengths of the alkyl chains of APG, it can be concluded that arachidyl glucoside and arachidyl behenyl alcohol are more lipophilic compared to cetearyl glucoside and cetearyl alcohol (35). Emulsions stabilized with arachidyl glucoside and arachidyl behenyl alcohol as emulsifier showed a visibly firmer consistency compared to emulsions stabilized with cetearyl glucoside and cetearyl alcohol as emulsifier (33). Similar to the interpretation of the HPLC results, the release of SP from emulsions with arachidyl glucoside and arachidyl behenyl alcohol as emulsifier was

expected to be lower, since a larger amount of SP was trapped inside the complex structure of lamellar liquid crystals compared to the emulsions stabilized with cetearyl glucoside and cetearyl alcohol. The statistically lower permeation observed for F2 compared to F3 supports this structural interpretation. Our results confirmed this expectation, considering that at the same time a larger amount of SP was released from emulsions stabilized with cetearyl glucoside and cetearyl alcohol as emulsifier. On the other hand, it is assumed that emulsions stabilized with arachidyl glucoside and arachidyl behenyl alcohol as emulsifiers can lead to extended release of incorporated SP, which could be very important in acne therapy.

Many compounds have been identified as penetration enhancers, such as nitrogen derivatives, fatty acids, alcohols, esters, sulfoxides, glycols, surfactants, and terpenes (53). Alpha hydroxy acids improve the transport of drug molecules through the skin by various mechanisms, such as distribution in lipid bilayers and disruption of their ordered structures, improvement of drug distribution in the *stratum corneum,* and formation of lipophilic complexes with drugs (54). A positive effect of GA on the penetration of azelaic acid was indicated, as well as on the combination of hydroquinone, tretinoin, and topical corticosteroid (16, 17). GA could also have a positive effect on the penetration of SP (log P = 2.78, Ph. Eur. 10). The addition of GA to both emulsions led to a faster and greater release of SP, indicating that it can be used as a penetration enhancer, in addition to its inherent activity against acne. The increase in the rate and amount of released SP due to the presence of GA was greater in emulsions stabilized with cetearyl glucoside and cetearyl alcohol as emulsifier, as expected, having in mind that the addition of GA leads to a slight thinning of the emulsions and partial release of SP from the structure of lamellar liquid crystals. Also, the pH value of the emulsions can affect the amount of released SP. After the addition of the GA, the pH value of the emulsions was adjusted to around 3.75. Due to the better stability of the emulsions with arachidyl glucoside and arachidyl behenyl alcohol as emulsifier in acidic environments, the release of SP from these emulsions would be slower compared to the emulsions with cetearyl glucoside and cetearyl alcohol as emulsifier (55). The average percentage dose depletion for all samples ranged from 11.49% in F2 to 23.51% in F3. These results are consistent with the fact that steady state release kinetics can typically be assumed under conditions when the dose depletion is less than 30% (56). The release profiles were fitted to zero-order, first-order, and Higuchi kinetic models (Table V).

Table V

Kinetic modeling of SP release

Formulation	Zero-order (R^2)	First-order (R^2)	Higuchi (R^2)
F1	0.973	0.876	0.995
F2	0.991	0.944	0.998
F3	0.994	0.921	0.999
F4	0.989	0.902	0.997

Among the tested models, the Higuchi model demonstrated the highest coefficients of determination (R^2 = 0.995 - 0.999) for all formulations, indicating diffusion-controlled drug release from the semi-solid matrix, which is typical for semi-solid preparations (57). Although zero-order kinetics also showed high linearity, the corresponding R^2 values were consistently lower, while first-order fitting exhibited the weakest correlation.

Besides GA, lactic acid (LA), which has a similar structure to GA, has the potential to be an effective percutaneous penetration enhancer in topical semisolids (15). It is believed that due to its structure, LA can interact with the components of the *stratum corneum* and, as a consequence, improve the permeation of drugs through the skin (15). Several studies have shown that LA is an effective penetration enhancer for some drugs that have similar structure and log P values as SP (indomethacin, oxybutynin, alprazolam, testosterone, estradiol, salicylic acid, dexamethasone, 5-fluorouracil, and ibuprofen lysine) in transdermal delivery systems (58-60).

Conclusions

SP, as an antiandrogen, is used topically to treat acne disorder, and literature data is supporting its safety and efficacy. This was the first study investigating SP content and release profile from topical emulsions, which is very important for a successful therapeutic outcome for this kind of preparation. We aimed to define the parameters of the HPLC method and solid-phase extraction that enable the SP content determination from emulsions stabilized with APG emulsifiers. We also wanted to investigate whether the presence of GA, which is used in acne therapy, would have a positive effect on the SP release profile from the emulsions. The HPLC method has proven successful for the analysis of SP content from emulsions stabilized with APG emulsifiers. The proposed method is successfully applied to the prediction of *in vitro* SP release from new topical emulsions using Franz diffusion cells. Also, we showed that SP release from the emulsions and permeation through the membrane depended on the

emulsifier and other components of the emulsion. The presence of GA in both emulsions led to a faster and greater release of SP, indicating that GA can be used as a penetration enhancer, in addition to its inherent activity against acne.

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Availability of data and materials

The data generated in the present study are included in the figures and/or tables of this article.

Ethics approval and consent to participate

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AI use disclosure

Not applicable.

Conflict of interest

The authors declare no conflict of interest.

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