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Formulation and investigation of innovative heterogeneous disperse systems

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1. Introduction and aims

In order to achieve sufficient therapeutic effect, the active substance must be delivered to the target organ in effective concentration. Except intravenous administration, drugs must first enter the bloodstream, from where they enter the extra- or intracellular space, to the site of action. In order to ensure this, we have to face a number of complex problems during the formulation of both external and internal preparations. The type of the pharmaceutical dosage form and the purpose of the formulation will be determined by several aspects depending on the route of administration.

Designing the most suitable pharmaceutical dosage from the pharmacon not only increases the absorption and bioavailability of the active substance, but also its therapeutic effect. During the formulation process of different innovative drug delivery systems, our main focus is on increasing the dispersity of the drug and its transport across different biological barriers.

In addition to protect our organs and tissues from the harmful physical, chemical and biological effects of the environment, the biological barriers of the human body play an essential role in maintaining the homeostasis of our body. However, these cell-connecting structures present a challenge for drug delivery, as the drug must cross both barriers and membranes to be absorbed into the bloodstream and transported to the site of action. One of the most important research area of pharmaceutical technology for both external and internal administration is enhancing the permeability of drug candidates across barriers. Using the appropriate penetration enhancing excipients during formulation can be the key to facilitate or even enhance the absorption of the active substances. Several scientific experiments have demonstrated that lipid-type surfactants can modulate trans- and paracellular transport, making them essential excipients for both external and internal drug delivery systems.

The formulation of micro- and nanoparticles from poorly water-soluble pharmacons can increase the dispersity, which leads to a decrease in particle size and an increase in specific surface area of the particles. This way we can increase the absorption of the active substance, and thereby the efficacy as well.

In our research, we choose those drug administration fields where effective drug delivery is the most challenging. In our experiments, we have formulated and investigated high-dispersity heterogeneous disperse drug delivery systems that support the successful feasibility

of the above-mentioned drug delivery strategy. We aimed to develop challenging external and internal formulations as well.

In the development of external dosage forms phytopharmacons are clearly one of the most challenging active ingredients. In the first part of our experiments, we formulated external dosage form containing Plantago lanceolata extract as active ingredient, a well-known plant in traditional medicine as well. In addition to the poor chemical stability of the active components with various polarities of the plant extract, their poor penetration through the skin greatly limits the applicability in pharmaceutical technology. Our aim was to formulate and investigate novel Plantago-loaded liquid crystal delivery systems. Besides the active pharmaceutical ingredient protective effect of the applied vehicle, the objective of our formulations were increased bioavailability.

In our attempts to develop an enternal dosage form, we also tried to face with the biggest challenges. In the second part of our experiments, we developed an innovative peptide formulation for oral administration containing melanin concentrating hormone (MCH) as active pharmaceutical ingredient. During formulation we had to overcome several obstacles resulting from peptides' unfavorable properties. Frequent peptide degradation in the GI tract, low epithelial permeability and amphiphilicity all block the way to the success of oral pharmaceutical formulations. Peptide-loaded calcium cross-linked alginate beads were formulated by a controlled gelification method in order to protect the model peptide. The incorporation of various permeation enhancers has been successfully enhanced oral bioavailability, therefore they can offer a successful strategy.

The experience gained in these two different fields contributes to the development of a drug formulation approach that can be used to address a wide range of therapeutic challenges. In my thesis, I describe the formulation process of a challenging external and a complicated oral dosage form.

2. Materials and methods

2.1. Materials

Gelucire 44/14, Lauroglycol 90, Labrasol, and Transcutol HP surfactants were kind gifts from Gattefossé (Lyon, France). Low viscosity grade sodium alginate was obtained from BÜCHI Labortechnik AG (Flawil, Switzerland). HaCaT cell line (human keratinocyte cells) was obtained from Cell Lines Service (CLS, Heidelberg, Germany), while human adenocarcinoma cancer cell line (Caco-2) was originated from the European Collection of Cell Cultures (ECACC, Public Health England, Salisbury, UK). MTT reagent 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, and buffer solutions such as Hank's Balanced Salt Solution (HBSS) and phosphate buffered saline (PBS), cell maintenance solutions, such as Dulbecco's Modified Eagle's Medium (DMEM), heat-inactivated fetal bovine serum (FBS), L-glutamine, a non-essential amino acids solution, and a penicillin-streptomycin solution were ordered from Sigma-Aldrich (St. Louis, MI, USA). Ninety six-well cell plates, twenty four-well cell plates and culturing flasks were obtained from VWR International (Debrecen, Hungary). A TBARS Assay Kit was bought from Cayman Chemical (MI, USA). 2,2-diphenyl-1-picrylhydrazyl (DPPH), absolute ethanol, IL-1β, and TNF-α were pur-chased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Formulation and investigation of liquid crystals containing Plantago Lanceolata extract

2.2.1. Dry Plantago lanceolata Leaf Methanolic Extract

Pharmacopoeial standard quality *P. lanceolata* leaves were purchased from commercial origin. Leaves were grinded and reduced to a finely divided powder for further application. The powder was extracted with MeOH under reflux (100 g Dry Weight DW- 400 mL MeOH) for 30 min, filtered, and evaporated to dryness in a rotary evaporator. The extract was defatted with hexane (3 × 50 mL).

2.2.2. Formulation of Liquid Crystals

To obtain the optimal concentration range of components for the boundary of liquid crystals, the pseudoternary phase diagram was constructed using the water dilution method. According to the toxicity screening evaluation, Labrasol, Lauroglycol 90, and Gelucire 44/14 were selected as surfactants. First, the required amount of *Plantago lanceolata* extract (equal

to 5% for each composition) was dissolved in the mixture of the surfactants. Then, water was added to the system under moderate stirring. The water content of the compositions varied from 37% to 56%, while the ratio of Labrasol/Lauroglycol 90 was constant (6:1). Four different formulations were selected for further investigation.

2.2.3. In Vitro Permeation Study

The dissolution rate of *Plantago lanceolata* extract from the formulated LC systems (PL-LC compositions I–IV) was evaluated using a vertical Franz diffusion chamber apparatus (Hanson Microette TM Topical and Transdermal Diffusion Cell System) in triplicate . Samples were applied as the donor phase on a synthetic cellulose acetate membrane previously soaked in isopropyl myristate. We used 30% (*v/v*) alcohol as the receptor phase and samples were stirred with a magnetic rotor at 350 rpm to enhance the dissolution rate of PL extract. The receptor solution was thermostated at 32 °C during the entire experiment to imitate the temperature of physiological skin on the Franz cell membrane. Samples of 1.0 mL of the receptor medium were sequentially collected at predetermined time points (every 15 min for 6 h) and replaced with fresh receptor solution after each sampling. Drug content was analyzed using a UV–Vis spectrophotometer (Shimadzu, Tokyo, Japan) at a wavelength of 517 nm.

2.2.4. Cell Culturing

HaCaT cells were maintained in Dulbecco's modified Eagle's medium (DMEM) in a plastic cell culture flask and supplemented with 2 mM L-glutamine, 100 mg/L gentamycin, and 10% heat inactivated fetal bovine serum at 37 °C in 5% CO_2 atmosphere. The culture medium was changed twice per week. The cells were routinely maintained by regular passaging. The cells used for cytotoxic and antioxidant experiments were between passage numbers 10 and 30. Keratinocytes were treated with a combination of the proinflammatory cytokines TNF-α (20 ng/mL) and IL-1β (25 ng/mL) according to Da Hee Choi et al. to obtain TNF-α-/Il-1β-induced human PSmHaCaT cells.

2.2.5. MTT Viability Assay

The cytotoxicities of applied excipients were evaluated using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay. HaCaT cells in complete medium were seeded on flat bottom 96-well tissue culture plate at a final density of 10^4 cells/well. After 7 days, the medium was removed, the cells were washed with PBS,

surfactant solutions were added, and the cells were incubated for a further 60 min. After removing the samples, a 3 h incubation with 100 μL MTT solution followed. The dark blue formazan crystals were dissolved in acidic isopropanol (isopropanol:hydrochloride acid = 25:1). The absorbance was measured at 570 nm against a 690 nm reference with FLUOstar OPTIMA Microplate Reader. Cell viability was expressed as the percentage of the untreated control.

2.2.6. Real-Time Cell Analysis and Transepithelial Electrical Resistance Measurements

Transepithelial electrical resistance (TEER) is a well-known and valuable method for in vitro barrier tissue integrity assessment. The measurement concept is relatively straightforward, with TEER measurements performed by applying an AC electrical signal across electrodes placed on both sides of a cellular monolayer and measuring voltage and current to calculate the electrical resistance of the barrier. In our investigation, 10^4 cells were seeded on special e-microplates as DMEM culturing medium was applied. For the PSmHaCaT cells, inflammation was induced by IL-1β and TNF-α blends, as described previously. Resistance values were measured using the xCELLigence RTCA instrument and data, such as the cell index, were automatically analyzed by the Integrated RTCA software. Observations were confirmed by traditional TEER measurements as well. Tran-sepithelial electrical resistance (TEER) was analyzed by seeding HaCaT and PSmHaCaT cells on 12-well Transwell inserts (Corning Transwell Clear, diameter: 6.5 mm, pore size: 3.0 lm) at a density of 2×10^4 cells/500 μL DMEM. TEER values were measured after 6 h of seeding and then measured every 12 h for 7 days with a Millipore Millicell-Ers 00.001 apparatus.

2.2.7. UV-C Exposure on HaCaT Cells

The UV protective effects of PL-LC compositions were investigated in HaCaT cells. Cells were maintained under the described conditions. HaCaT and PSmHaCaT cells were seeded at the density of 10^4/200 μL on 96-well plastic plates. The test was performed on the 5th day of their proliferation (when the cells did not reach the total confluency state according to our RTCA measurements). The compositions were changed by replacing water with an equal volume of DMEM cell culture medium to avoid the harmful effect of the water. Each cell line was tested with and without the compositions by UV exposition performed by Sol-UV-4 UV Solar Simulator (Oriel® USA). For the first experiment, we worked with the PL-LC formulations. In this experiment, we had two groups of HaCaT and PSmHaCaT cell lines. We

applied the UV stimulus only to the second group. As a follow-up, all cell groups were tested directly after the exposure (or after the same six minutes later in case of control group), then 12, 24, and 48 h later via the MTT cell viability assay. In the second part of this experiment, cells were incubated with 0.5% v/v PL-LC compositions for 24 h (from their 4th day in proliferation), then exposed to UV-induced oxidative stress for 6 min. HaCaT cell viability was measured directly after the exposure by MTT assay. The follow-up investigation was carried out for 48 h, as in the previous case.

2.2.8. Antioxidant Assays

2.2.8.1. DPPH Radical Scavenging Activity

A DPPH reduction assay was performed to screen the scavenger activity of the liquid crystal formulations containing Plantago lanceolata. DPPH is a stable free radical that is capable of changing color in the presence of different antioxidants. The control solutions were the appropriate blank compositions and a 5% v/v water dispersion of PL extract (CE). Each sample containing 5% v/v PL extract was reacted with the stable DPPH radical in ethanol (96%). The reaction mixture consisted of adding 100 µL of sample, 900 µL of ab-solute ethanol, and 2 mL of DPPH radical solution (0.06 mM) in absolute ethanol. The mixtures were incubated for 30 min. When DPPH reacted with an antioxidant compound that can donate hydrogen, it was reduced. The reaction resulted in a color change from deep violet to light yellow. Quantitative measurement of the remaining DPPH was carried out with a UV spectrophotometer (Shimadzu Spectrophotometer, Tokyo, Japan) at a wavelength λ of 517 nm. In the case of photometric determination mixtures, absolute eth-anol served as the background. The control solutions were the same compositions without P. lanceolata extract. To demonstrate the improved antioxidant effect of the combinations, blank P. lanceolata extract (5% v/v) was applied as well. The scavenging activity percentage (AA% = antioxidant activity) was determined according to Mensor et al.:

$$AA\% = 100 - [((Abs_{sample} - Abs_{blank}) \times 100)/Abs_{control}].$$

2.2.8.2. Lipid Peroxidation (MDA) Assay

Reactive oxygen species can generate the lipid peroxidation process in an organism. Malondialdehyde (MDA) is one of the final products of polyunsaturated fatty acids pe-roxidation in the cells. An increase in free radicals causes overproduction of MDA. In the lipid

peroxidation assay protocol, the MDA in the sample reacts with thiobarbituric acid (TBA) to generate an MDA–TBA adduct. Cells (1×10^6) were homogenized on ice in 300 µL of MDA lysis buffer (with 3 µL BHT (100×), then centrifuged (13,000 ×g, 10 min) to remove insoluble material. An aliquot of 200 µL of the supernatant was analyzed for lipid perox-idation with a TBARS Assay Kit (Cayman Chemical). Lipid peroxidation was determined through the reaction of malondialdehyde (MDA) and thiobarbituric acid (TBA), forming a colorimetric (532 nm) MDA–TBA adduct.

2.3. Formulation and investigation of peptide loaded sodium-alginate beads

2.3.1. Preparation of Sodium-Alginate and $CaCl_2$ Solutions

A total of 1.50% (*w*/*v*%) polymer solution was prepared by dissolution of 3.30 g of low viscosity grade sodium-alginate in 200 mL distilled water. The solution was mixed for 180 min at room temperature (25 °C) under vigorous stirring (300 rpm) to obtain a homogeneous solution. For the $CaCl_2$ solution (100 mM), 14.70 g calcium chloride dihydrate was dissolved in 1000 mL deionized water.

2.3.2. Preparation of Peptide-Loaded Alginate Beads

The MCH-loaded alginate microbeads were prepared by a controlled polymerization method using a Büchi Encapsulator B-395 Pro apparatus. The peptide was finely distrib-uted in 40 mL of 1.50% sodium-alginate solution combined with the 0.01 v/v% of penetra-tion enhancers (Labrasol, Transcutol HP). Mixtures were loaded into a 40 mL BD Lu-erLock type syringe. The polymer–pharmacon mixture was forced into the pulsation chamber by a syringe pump at speed 5.00 mL/min. The solution was passed through an electrical field between the nozzle (80 µm, 200 µm, 1000 µm). A 1000 V set electrode separated the alginate solution into equal size droplets by 1800 Hz frequency. The alginate beads were left to harden for 15 min in calcium-chloride solution. The finely di-vided particles were washed with hardening solution and filtered on a 0.4 µm pore size membrane by a vacuum pump and freeze dried for 24 h at −110 °C.

2.3.3. Encapsulation Efficiency

To determine the encapsulated drug content in the beads, a 1 mL sample was meas-ured from 100 mM of the calcium-chloride hardening solution right after formulation. Drug

concentration was determined by radioimmunoassay (RIA). Encapsulation effi-ciency (EE) was calculated by the following equation:

$$EE = \left(\frac{TQ - HQ}{TQ}\right) \times 100$$

where *HQ* is the drug quantity present in the hardening solution, and *TQ* is the theoretical drug content in the beads.

2.3.4. Study of Swelling Behavior

The swelling behavior of the alginate spheres was determined gravimetrically. A total of 85 mg dry beads were weighed and soaked in 100 mL distilled water at room temperature. The beads were removed after 1 h, and the excess wetness was eliminated with vacuum filtration. The equilibrium water uptake was calculated using the following equation:

$$EWU = \left(\frac{Ws - Wd}{Ws}\right) \times 100$$

where *Ws* is the weight of swollen beads and *Wd* is the initial weight of the dry beads.

2.3.5. Scanning Electron Microscopy

Electron microscopic analysis of the shape, size and surface area of the particles was performed using a Hitachi Tabletop microscope (TM3030 Plus) (Hitachi High-Technologies Corporation, Tokyo, Japan). Samples were attached to a plate covered with double-sided adhesive tape. An accelerating voltage of 5 kV was used during mi-crography.

2.3.6. Particle Size Distribution

Particle size distribution of the beads formulated with a 200 μm nozzle was per-formed with a HoribaPartica LA-950V2 (Horiba, Ltd., Kyoto, Japan) laser diffraction particle size analyzer, operated in wet mode with distilled water (1000× dilution), performing at least three parallel measurements on each sample.

2.3.7. In Vitro Dissolution

The rotating paddle method by Erweka DT 800 apparatus (Erweka GmbH, Langen, Germany) was applied to characterize drug release from the particles. In vitro dissolution was

evaluated as 400 mg of the formulated dry beads were placed in 100 mL buffer phosphate solution (pH = 6.80) at 37 °C. A total of 1000 µL of the dissolution media was collected at determined time intervals (0., 5., 15., 30., 60., 90., 120. min). The drug concentration in each sample was analyzed by radioimmunoassay.

2.3.8. Enzymatic Stability

Enzymatic degradation in the presence of proetolytic enzymes pepsin and pancreatin was investigated. Peptide-loaded particles were added to 100 mL of artificial gastric fluid (simulated gastric fluid - SGF) with pepsin, or artificial intestinal fluid (simulated intestinal fluid - SIF) with pancreatin and incubated at 37 °C under moderate stirring at 100 rpm. SGF and SIF were prepared as per European Pharmacopoeia specifications. A total of 1000 µL samples were collected at determined time intervals for 120 min, and an equal volume of ice-cold reagent was added to stop the enzymatic reaction (0.10 M NaOH for SGF and 0.10 M HCl for SIF). Samples were analyzed by radioimmunoassay.

2.3.9. Cell Culturing

A Caco-2 immortalized human adenocarcinoma cell line was purchased from The European Collection of Cell Cultures (ECACC). Cells were seeded and maintained in plastic cell culture flasks in Dulbecco's Modified Eagel's medium (DMEM Sigma-Aldrich Ltd., Budapest, Hungary). Cell culturing media was supplemented with 2 mM L-glutamine, 100 mg/L gentamycin (Sigma-Aldrich Ltd., Budapest, Hungary) and 10% heat inactivated fetal bovine serum (FBS) (Sigma-Aldrich Ltd., Budapest, Hungary). Cells were stored in incubators at 37 °C in a 5% CO_2 atmosphere. The colon epithelial Caco-2 cell line forms a single cell layer with well-defined tight junctions.

2.3.10. Cell Viability Assay

Caco-2 cell viability was evaluated using the MTT method. Cells were harvested and seeded at a density of 10^4 cells/well on flat-bottom 96-well tissue culture plates. Cells were allowed to grow for 7 days under the abovementioned conditions. For the viability measurements, the DMEM was removed. Cells were incubated with the applied penetration enhancer (Labrasol, Transcutol HP; Gattefossé, Saint-Priest, France) solu-tions, all other components and blends for 30 min. Samples were removed and cells were washed with buffer solution. Mitochondrial activity of the cells was determined after 3 h incubation with MTT at the

concentration of 0.50 mg/mL. Dark blue formazan crystals were dissolved in acidic isopropanol (isopropanol:1.00 N hydrochloric acid = 25:1). The absorbance of dissolved formazan crystals correlates with the ratio of the living cells. Ab-sorbance was measured with a FLUOstar OPTIMA Micro-plate Reader (BMG LABTECH, Offenburg, Germany) at 570 nm against a 690 nm reference. Cell viability was demon-strated as the percentage of the untreated control.

2.3.11. Permeability Test

A human colon adenocarcinoma cell line was used as the model of human intestinal absorption of MCH. Caco-2 cells were seeded on 12-well ThinCert™ transwell polyester inserts (pore size: 0.40 μm; Greiner Bio-One International, Mosonmagyaróvár, Hungary) at a density of 2 × 105 cells/cm2 and grown for 21–28 days. Transepithelial elec-trical resistance (TEER) was measured every 2 days. Measurements were begun when TEER values of the inserts reached 1000 Ω × cm2 value. The culture medium in the apical and basolateral compartments was replaced with HBSS and the monolayers were pre-incubated for 30 min at 37 °C. After that, the experimental solution was pipetted to the apical chambers. In transport experiments, we have studied the permeability of different types of samples; one containing only peptide, another with the same amount of MCH containing 0.01% Labrasol and/or 0.01% Transcutol HP as a penetration enhancer. A total of 300 mg of the samples were dissolved in 60 mL of PBS buffer for 60 min. As a negative control HBBS was examined. The permeated amount of MCH was determined after 60 min. These samples were collected from the basolateral compartment and replaced with fresh HBSS. Samples were analyzed by radioimmunoassay. To complete the investigation, membrane function was monitored by transepithelial electrical resistance measurements.

2.3.12. Transepithelial Electrical Resistance Measurements

Transepithelial electrical resistance was measured with Millipore Millicell-ERS 00001equipment (Waltham, MA, USA). During the transmembrane experiments, TEER values were determined every 15 min. As a follow-up, measurements were contin-ued in the following 24 h to investigate the recovery.

2.4. Statistical analysis

Data were handled and analyzed using Microsoft Excel 2013 and SigmaStat 4.0 (version 3.1; SPSS, Chicago, IL, USA, 2015), and herein presented as means ± SD. Comparisons of

results were performed with one-way ANOVA followed by Tukey's range test. All experiments were carried out in triplicates, difference of means was regarded as significant in the case of $p < 0.05$.

3. Results

3.1. Formulation and investigation of liquid crystals containing Plantago Lanceolata extract

3.1.1. Dry Plantago lanceolata Leaf Methanolic Extract

After the procedure, 100 g of plant drug gave 25.2 g PL extract. The extract was chemically characterized with the LC-ESI-MS method, after calibration with authentic standards. The extract contained 5.99% acteoside, 2.34% aucubin, and 1.21% catalpol, equivalent to 1.51%, 0.59%, and 0.31% on plant material dry weight, respectively.

3.1.2. Formulation of Liquid Crystals

The aim of the pseudoternary phase diagram construction was to determine the occurrence range of the liquid crystals. To study the effect of water content on the liquid crystal structure, samples with different water contents (10–90%) were prepared. Mainly during the titration, microemulsions formed at various pro-portions of components. When the ratio of water was increased to above 90%, macro-emulsions began to form, regardless of the proportion of Gelucire 44/14 and Labra-sol/Lauroglycol 90 surfactants. In contrast, liquid crystal systems formed only at a certain, tighter range of the components when the mix contained Labrasol/Lauroglycol 90 surfac-tants under 10%. Four different compositions were selected according to the phase dia-gram with 6:1 constant ratio of Labrasol/Lauroglycol 90. Our systems were stable and clear in the Labrasol/Lauroglycol 90 range of 4.1–9.9% and the Gelucire 44/14 range 31.1–52%.

3.1.3. In Vitro Permeation Study

We ranked the release of drug from the four compositions in the following descending order: PL-LC 2 > 1 > 4 > 3. Our results showed that the drug release was better from the compositions that contained more of the surfactant pair Labrasol and Lauroglycol 90. The best diffusion was achieved from compositions I and II, in which the surfactant range was 9.9% and 7.8%, and the diffused amount of PL extract was over 50%. In the case of compositions III and IV, which contained less of the surfactants, worse drug diffusion occurred, as the released drug was under 40%.

3.1.4. Assessment of HaCaT Cell Proliferation

Proliferation tests were conducted on HaCaT cells seeded at 10^4/200 μL density using an xCELLigence RTCA analyzer. However, impedance-based determination of HaCaT cell proliferation demonstrated an obvious increase in the cell index in each of the cases, and the kinetics of the proliferation showed differences. Whereas the native HaCaT cells reached the confluent monolayer state after seven days, the IL-1β- and TNF-α-stimulated keratinocytes (PSmHaCaT) reached the plateau phase earlier after rapid proliferation. Moreover, PSmHaCaT keratinocytes demonstrated higher cell index values at this phase. To confirm this conclusion, classic transepithelial electrical resistance (TEER) measure-ments were also performed. The result of the transepithelial electrical resistance (TEER) measurements agreed with the RTCA tests. Higher TEER values were observed in PSmHaCaT cells than in native keratinocytes at the same time.

3.1.5. MTT Viability Assay

MTT cytotoxicity tests were performed on HaCaT cell monolayers to ensure safe application. The cytotoxicities of the formulated blends were evaluated in different concentrations. Each component of the compositions was also tested. The cell viability was less than 50% compared to that of the negative control (phosphate buffered saline (PBS)), even in a high concentration. We found that all compositions were tolerated well. There were no significant differences between LC- and PBS-treated (negative control) groups, although we concluded that composition II decreased cell viability the least.

3.1.6. DPPH Radical Scavenging Activity

The radical scavenging activity assay is based on the ability of a stable free radical (DPPH) to change color in the presence of antioxidants. The antioxidant capacities of LC compositions I–IV, with or without PL extracts, were tested. As controls, the appropriate blank LC formulations and water dispersion of 5% PL extract (CE) were applied. As previously described, formulated LC samples also contained 5% v/v PL extract. The percentage of antioxidant activity (AA%) of each substance was assessed using a DPPH free rad-ical assay. The DPPH radical scavenging activity was measured according to the methodology described by Brand-Williams et al. According to our results, we concluded that there were significant differences between LC-PL-composition-treated groups, compositions without PL- and PL-composition-treated groups, and the CE-treated positive control sample. The performed radical

scavenging activity assay demonstrated that all compositions were able to significantly inhibit DPPH mean oxidation. By distinguishing the tested LC-PL-compositions, we determined that compositions I and II were the most effective and composition IV showed the least activity, although its antioxidant capacity was found to be significantly higher than that of the nonformulated PL extract (CE).

3.1.7. Lipid Peroxidation (MDA) Assay

The protective effects of PL-LC compositions I–IV against UV irradiation were examined on HaCaT and PSmHaCaT cell lines. Evaluated MDA level differences confirmed not only the efficiency of PL extract as an antioxidant complex, but also the importance of the appropriate components of the formulation. Besides all PL-LC compositions being able to decrease MDA levels compared to the untreated and unformulated PL extract controls, we found that PL-LC composition I (with the highest penetration enhancer content) significantly decreased the MDA level.

3.1.8. UV-C Exposure on HaCaT Cells

The effect of LC-PL compositions against UV-C irradiation was determined on Ha-CaT and stimulated keratinocyte (PSmHaCaT) cell lines. Compositions were changed by replacing water with an equal volume of Dulbecco's modified eagle's medium (DMEM) cell culture medium (Table 2) to avoid the adverse effects of water. As in the first experiment, no PL-LC treatment was applied. These results demonstrated the destructive effect of UV-C radiation on the different cell lines and revealed that 6 min of UV-C exposure resulted in a significant decrease in viability. To evaluate the UV-C-stimulated proliferation changes, after the UV-C exposure, MTT follow-up measurements were performed at the 12, 24, and 48 h time points. We concluded that 6 min of UV-C exposure resulted in robust PSmHaCaT cell pro-liferation. After two days, cell viability of UV-C-stimulated PSmHaCaT cells transcended the viability of untreated cells. Figure 9 shows the cell viability of the PL-LC treated cells after 6 min of UV-induced oxidative stress. Data evaluation showed that PL-LC treatment partly blocked the harmful effect of UV-C radiation. Besides the antioxidant effect of PL extracts, the penetration-enhancing effect of Labrasol/Lauroglycol 90 prevailed as a larger amount of drug entered the cells per unit time from compositions I and III containing more of the surfactant pair, thus retaining cell viability. Comprehensive analysis of fol-low-up experiments demonstrated that

LC-PL treatment was not able to protect the keratinocytes, but the observed hyperproliferation of PSmHaCaT cells caused by UV-C exposure was avoided.

3.2. Formulation and investigation of peptide loaded sodium-alginate beads

3.2.1. Encapsulation Efficiency

Encapsulation efficiency measurements resulted in at least 56%. The lowest EE was calculated in the case of simple MCH beads. Labrasol and Transcutol HP supple-mented beads showed the highest EE although no significant differences had been evaluated between the closing ability of the compositions.

3.2.2. Study of Swelling Behavior

Bead swelling behavior investigation results show that the value of equilibrium water uptake depends on the particle size of beads, as the smaller is the diameter and thus the larger is the specific surface area, the higher is the water uptake capacity. These data might be useful during the formulation since swelling behavior influences the applicability.

3.2.3. Scanning Electron Microscopy

The surface morphology of the represented microspheres showed spherical shaped beads having some squashes on the surface, probably due to the drying process. The SEM observation also demonstrated that the diameter of microbeads is consistent with the results of particle size distribution.

3.2.4. Particle Size Distribution

Microbeads were analyzed by a HoribaPartica LA-950V2 laser diffraction particle size analyzer. According to the volume moment mean values, the particle size of the formulated microbeads is close to the theoretical 200 μm.

3.2.5. In Vitro Dissolution

In vitro peptide release experiment was conducted in buffer phosphate solution (pH = 6.80). Samples were tested by RIA. Dissolution profiles were determined by plotting the experimental data. According to the data presented, a biphasic release pattern can be observed. MCH was released in a prolonged manner, since in the first 60 min no significant release could

be measured. After one hour, an initial burst release started, where 68.30% of the incorporated MCH was released from the beads. The release ratio of MCH after 120 min was significantly low.

3.2.6. Enzymatic Stability

It had been shown that only 3.00 ± 0.50% and 8.00 ± 0.50% active MCH could be measured after 30 min incubation in SGF and SIF, respectively, form the free (not formulated) MCH samples. Peptide was completely degraded after 1 h incubation in SGF, while only 1.00 ± 0.50% MCH recovery occurred after 2 h incubation with SIF. Our measurement demonstrated that bead formulations are able to protect the model peptide. In case of each formulation at least 70% of MCH was protected from SGF and SIF degradation.

3.2.7. MTT Viability Assay

According to the performed cell viability measurements it was demonstrated that all of the selected excipients are safe in the applied concentration. Although concentration dependent toxicity can be observed, sodium-alginate and calcium-chloride dihydrate did not show relevant toxicity, not even at higher concentrations. Our results demonstrates that penetration enhancers showed obvious toxicity at higher concentrations; thus, IC_{50} values were evaluated. IC_{50} values of the excipients were determined as Labrasol 0.41 (v/v%), Transcutol HP 0.37 (v/v%) and 1:1 ratio blended Labrasol:Transcutol HP composition 0.35 (v/v%) in our experiments. The formulated drug delivery systems with 0.01 (v/v%) of penetration enhancer content did not resulted in disadvantageous changes to the monolayer.

3.2.8. Permeability Test

Before the permeability test, Caco-2 monolayers on the inserts showed high (600 $\Omega x cm^2$) TEER values, indicating tight barrier properties. The permeability of MCH encapsulated in alginate beads was significantly higher than that for MCH solution. An increased MCH permeability was measured in the presence of the penetration enhancer, suggesting the opening of a paracellular pathway.

3.2.9. Transepithelial Electrical Resistance Measurements

The membrane integrity of adenocarcinoma cells was measured using TEER measurements. After 30 min, compositions caused a significant decrease of transepithelial

electrical resistance. The follow-up measurements demonstrated that TEER values increased in fresh DMEM after 60 min. TEER values of MCH beads-treated monolayers increased immediately after the treatment. Moreover, transepithelial electric resistance values increased above 90% of the baseline at the end of experiment.

4. Discussion

4.1. Formulation and investigation of liquid crystals containing Plantago Lanceolata extract

The importance of the role of phytopharmacons in external therapy continues to increase due to the useful chemical substances they contain. Although their advantageous use is proven, the effectiveness of these compounds needs to be improved by pharmaceutical formulation techniques. In topical drug delivery we have to face with two major challenges. Given the fact that patient compliance is quite low in topical drug therapy, besides drug solubilisation and adequate penetration through the stratum corneum, patient adherence is extremely important. The effect of phytopharmacons can be enhanced effectively by various innovative formulations containing penetration enhancers. The aim of the first part of our research was to formulate safe dosage forms containing Plantago lanceolata extract with enhanced cutaneous drug delivery. The effectiveness of liquid crystal systems in local therapy has been described previously. Their structure and chemical properties are similar to the cell membrane, thus providing and facilitating drug penetration through the stratum corneum. In recent years, they have received considerable attention as potential drug delivery systems due to their ability to form a sustained release drug matrix protecting the drug against both physical and chemical degradation. Liquid crystals are anisotropic materials that demonstrate both liquid- and solid-like properties and the capability to solubilize both oil- and water-soluble compounds. Moreover, liquid crystal compositions can be synthesized to form valuable surfactants with a defined penetration enhancer effect. Due to these properties, these systems are advantageous not only from the formulation point of view, but also for patients in their everyday lives. Formulated liquid crystal compositions with an appropriate ratio of Labrasol/Lauroglycol 90 (6:1)/Gelucire 44/14/water were selected using the structured pseudoternary phase diagram, and the percentage of diffused PL extract was evaluated. To evaluate the behavior of the formulated LC systems, a wide variety of in vitro cell line assessments were performed. Besides the HaCaT cell line, TNF-α- and Il-1β-induced human PSmHaCaT cells were used. Impedance-based determination of HaCaT cell proliferation demonstrated an obvious increase in the cell index in each case. Moreover, the kinetic of the proliferation showed differences, i.e., whereas the native HaCaT cells reached the confluent monolayer state after seven days, the IL-1β- and TNF-α-stimulated keratinocytes (PSmHaCaT) reached the plateau phase earlier after rapid proliferation. Furthermore, PSmHaCaT keratinocytes demonstrated higher cell index values at this phase. The performed MTT tests confirmed that neither the formulated LC systems nor their components considerably

affected cell viability. Thus, all the formulated compositions were safe. The radical scavenging activity assay demonstrated that the formulated compositions were able to significantly inhibit mean DPPH oxidation. By distinguishing the tested LC-PL compositions, compositions I and II proved to be the most effective and composition IV showed the least activity. The antioxidant properties of the PL-LC systems were evaluated with an MDA lipid peroxidation assay as well. Besides all the PL-LC compositions being able to decrease MDA levels compared to the untreated and unformulated PL extract controls, it was revealed that PL-LC composition I significantly decreased the MDA level. These results agree with the conjecture that penetration enhancers play a role in antioxidant efficiency. This observation was confirmed by the results of the performed UV-C irradiation assessments. Our results demonstrated the destructive effect of UV-C radiation on the different cell lines. The assessment revealed that six minutes of UV-C exposure resulted in a significant viability decrease. To broaden our knowledge after the UV-C exposure, MTT follow-up measurements were performed. These results showed that six minutes of UV-C exposure resulted in robust PSmHaCaT cell proliferation. After two days, the cell viability of UV-C-stimulated PSmHaCaT cells exceeded that of untreated cells. PL-LC treatment unequivocally partly blocked the harmful effect of UV-C radiation. Besides the antioxidant effect of PL extracts, the penetration-enhancing effect of Labrasol/Lauroglycol 90 prevailed as a larger amount of drug entered the cells per unit time from compositions I and III containing more of the surfactant pair, thus retaining cell viability. Moreover, it was revealed that LC-PL treatment was not even able to protect the keratinocytes, but the observed hyperproliferation of PSmHaCaT cells caused by UV-C exposure was avoided. Our findings highlight the importance of surfactants playing a crucial role in improving efficiency, therefore, their proper selection is a highly important factor. We also found that the formulated PL-LC compositions provided a preferable option for skin protection against UV radiation.

4.2. Formulation and investigation of peptide loaded sodium-alginate beads

The rapid progress in biotechnology over the past few decades has accelerated the large-scale production of therapeutic peptides and proteins, making them available in medical practice. At this point, injections mean the most common way for administering proteins and peptides despite the numerous disadvantages, so the importance of alternative routes of peptide administration is not questionable. Despite inoculated peptides having saved millions of lives, their invasive nature has led to significantly reduced patient compliance in long-term therapy. Formulation of orally administered peptide-loaded drug delivery systems could be a solution to the above mentioned problem, however, development of oral protein delivery systems has been a persistent challenge to pharmaceutical technology due to their unfavorable properties. During formulation, we have to face with several obstacles in order to achieve adequate oral bioavailability. Proteolytic enzymes and effective barriers in the gastrointestinal tract can significantly limit the absorption of peptides and proteins. To overcome these problems several approaches are mentioned in the literature, such as enzyme inhibition, chemical modification, increasing membrane permeability or designing innovative carrier systems. The encapsulation of peptides into finely divided bioadhesive polymer carrier systems is a preferable and widespread method to improve oral bioavailability of peptide drugs due to their beneficial properties. In the second part of our research our aim was to develop and characterize novel melanin concentrating hormone (MCH) formulations for oral administration. The peptide-loaded calcium cross-linked alginate beads were formulated by a controlled gelification method in order to protect the model peptide. The great advantage of the compared to previous developments is that it allows formulation at room temperature, thus protecting the active ingredient from possible denaturation. Our measurements stated that the value of the equilibrium water uptake depends on the particle size of beads. Due to the applicability, a 200 μm diameter was found to be ideal. Performed SEM and DLS measurements confirmed that the morphology is appropriate due to the expectations and the particle size of the formulated microbeads being close to the theoretical 200 μm. Performed measurements demonstrated that the applied alginate-bead formulation method resulted in at least 56% encapsulation efficiency of MCH. Blended penetration enhancers did not affect the closing ability. We can state that our formulations are stable environments to encapsulate peptide drugs. Moreover, these compositions could successfully shelter the peptide from the harsh environment of the simulated conditions of the gastrointestinal tract according to our enzyme resistance tests. Peptide release tests demonstrated that during the first hour, 68.30% of incorporated MCH was

disengaged. Due to biosafety having a high priority in the case of novel pharmaceutical formulations, the toxicity of the selected excipients and compositions was evaluated by various in vitro methods. Our results confirmed that the formulated compositions are safe due to the fact that the compositions did not cause disadvantageous changes to the Caco-2 layers. Moreover, during our transmembrane permeability tests, it was demonstrated that the permeation of alginate-encapsulated MCH was significantly higher than native MCH solution. Moreover, an increased MCH permeability was measured in the presence of a penetration enhancer, suggesting the opening of paracellular pathway. To exclude the possibility of membrane damage in the background of this result, transepithelial electrical resistance follow-up measurements were performed. These results indicate that the barrier properties of the monolayers completely recovered within 12 h after penetration enhancer treatment. Our transepithelial electrical resistance measurements proved that the permeation enhancement effect of Labrasol and Transcutol HP did not lead to an irreversible disruption of the Caco-2 monolayers. These results suggest that the alginate beads are promising tools to protect peptide derivatives against GI degradation. Punctiliously selected excipients are able to ensure the biosafety and the appropriate physical properties. Prudent selection of penetration enhancers might lead to improved absorption by reversible alteration of barrier functions. These formulations can be vital tools for novel peptide delivery systems with excellent pharmaceutical properties. Moreover, they are able to improve patient's adherence and experience. According to the results, we managed to show that alginate beads are able to protect melanin concentrating hormones during the formulation. Moreover, the peptide was protected against gastric and intestinal enzymatic degradation as well. The combination of alginate carriers with amphiphilic surfactants has not been described yet. Our results demonstrated that these penetration enhancers might play a key role in bioavailability improvement.

5. Conclusion

In the first part of our work Plantago lanceolata loaded liquid crystal delivery systems were formulated for external use with the help of titration method. According to the results of in vitro permeation study, we found that the penetration enhancing excipients increase the penetration of the drug in a concentration-dependent manner. The results of DPPH and MDA antioxidant assays also confirmed the role of penetration enhancing excipients in antioxidant activity. Tests on the UV-C protection of the formulations demonstrated that the treatments clearly blocked some of the harmful effects of UV-C radiation. Although the treatments could not fully protect keratinocyte cells against radiation, our compositions significantly reduced the UV-C-induced hyperproliferation of TNF-α and IL-1 induced cells. Our experimental results have confirmed that surfactants play a crucial role in transdermal absorption of phytopharmacons, therefore their proper selection is a major factor to increase efficacy.

In the second part of our experiments, we designed innovative solid drug delivery systems for oral administration to increase oral bioavailability of peptide-type drugs. Peptide-loaded calcium cross-linked alginate beads were formulated from melanin-concentrating hormone by controlled gelification method in order to protect our model peptide. During preformulation, we successfully calibrated the instrument components for the formulation and also optimized the critical parameters for reproducible bead shape and size. Investigation of the physical properties of the beads confirmed that their shape and size are both appropriate. Based on the results of the encapsulation efficiency measurements, we concluded that the excipients have no effect on the amount of the entrapped drug. The results of permeability studies confirmed that from the formulations containing lipid-type surfactant excipients are able to penetrate more into the basal fluid across the synthetic membrane, so their presence enhances absorption in the intestinal tract. Based on the results of enzymatic stability evaluation, the formulated drug delivery systems successfully protected the model peptide against the harsh enzymatic conditions of the gastrointestinal tract. Our results confirm that using different amphiphilic surfactants can play a key role in enhancing oral bioavailability of peptide-type drugs.

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List of publications related to the dissertation

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