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Cyclodextrin-based nanoparticles for drug encapsulation

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Background

The use of nanocarriers for drug delivery represents nowadays a promising approach to overcome a series of pharmacological shortcomings of drugs, like low target specificity, rapid clearance, poor pharmacokinetics, severe side effects, and multidrug resistance phenomena.

Cyclodextrin-based nanoassemblies as drug carriers

Supramolecular cyclodextrin-based nanoassemblies mediated by host-guest interactions have gained increased popularity because of their "green" and simple preparation procedure, as well as their versatility in terms of inclusion of active molecules (Gref et al., 2012). Here we show that original nanoparticlesof around 100 nm are spontaneously prepared in water, by a lock and key mechanism involving formation of inclusion complexes between CDs on one water-soluble polymer and hydrophobic side chains on another one (Anand et al., 2012; Battistini et al., 2008; Daoud-Mahammed et al., 2009; Fraix et al., 2013; Othman et al., 2011). Whatever the mixing conditions, nanoparticles with narrow size distributions arespontaneously formed upon the contact of two polymeric aqueous solutions. Advantageously, the production of the nanoparticles can be scaled-up using a microfluidic device. In situ size measurements helped understanding the mechanisms involved in nanoparticle self-assembly. Individual nanoparticle tracking analysis enabled to establish that despite the non-covalent nature of the nanoassemblies, they were remarkably stable, even upon extreme dilution (few ng/mL). Contrast agents bearing adamantly moieties were incorporated into the nanoassemblies, showing high relaxivities (Battistini et al., 2008). Spectroscopic and photophysical studies helped understanding the interactions involved in the nanoassemblies loaded or not with drugs (Anand et al., 2012). Finally, another set of examples shows the utility of the nanoassemblies for bimodal anticancer phototherapy (Fraix et al., 2013).

Conclusion

This study paves the way towards the "green" scalable formation of supramolecular assemblies with potential uses in cosmetics and drug delivery.

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Geomaterials in the design of new drug delivery systems

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Introduction

The lecture is intended to provide an overview of the use of geomaterials in medical and health care applications, including their use as active pharmaceutical ingredients and classical excipients together with new applications in drug and gene delivery, tissue engineering, as well as prospective used in future therapeutics and diagnostics trends. Both natural geomaterials and their derivatives are attracting growing attention in a variety of fields and in particular as biomaterials. Pharmaceutical technology is not an exception and geo-source excipients are competing well with the synthetic materials.

Pharmaceutical uses of geomaterials: from conventional to innovative

For thousands of years, humans have been using available substances to achieve specific functions, including their use in contact with living tissues, organisms or micro-organisms. Like most of the materials used by humans, geomaterials are widely mentioned in the literature, and occasionally exploited clinically, as such, as devices or as part of devices to treat trauma and diseases. These classical used of geomaterials in health care applications will be revised.

In recent decades, research in biomaterials and their use in healthcare have increased all over the world. Applications of biomaterials in human healthcare include the development of new medical devices and prostheses, tissue engineering, bone regeneration, implants and surgical tools, diagnostic techniques, bioadhesives, artificial organs and drug delivery (Larsson et al., 2007). Biomaterials for medical applications must be biocompatible, and either bioresorbable or biodurable. Nanotechnologies and inorganic–organic hybrid technologies are currently two of the most active approaches to achieve these requirements. Many organic–inorganic hybrid materials and nanotechnology based targeted drug delivery systems imply the use of geomaterials.

Arrangement, structure and properties of pharmaceutically interesting geomaterials

Some geomaterials, and significantly carbonates (and carbon structures), phosphates and silicates (special clays and zeolites) induce no adverse effect in contact with a living organism (biocompatibility) and their presence may be beneficial (bioactivity) or without any significant health effect (bio-inert) but with biodurable properties. The specific function that a geomaterial have in any formulation depends on its composition, structure and properties, including both physical properties (particle size and shape, specific surface area, texture,…) and chemical features (surface chemistry, charge,…).

Calcium phosphate and calcium carbonate are natural nanosized geomaterials than can be used in drug delivery and tissue engineering. Calcium phosphate nanoparticles can be tailored to be biocompatible and show high adsorption capacities to be used in controlled and targeted drug release.

It must be also remarked all the possibilities in medicine of graphitic forms deriving from graphene as fullerenes, carbon nanotubes and graphite. The possibilities of these carbon materials in health care products are a new frontier of pharmaceutics (Hong et al., 2015; Pan et al., 2012; Sánchez et al., 2012).

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Calcium carbonate appears as three different anhydrous crystalline polymorphs: calcite, aragonite, and vaterite. Under standard conditions for temperature and pressure, calcite is the stable phase, while aragonite and vaterite are the metastable forms that readily transform into the stable phase. Vaterite and other polymorphic forms of carbonate are receiving great attention as nanocarriers in drug delivery.

Silicates, including some tectosilicates, as zeolites (Cerri et al., 2016), but mainly phyllosilicates (clay minerals) have also featured in pharmaceutics due to their adsorptive and ion exchange properties (Aguzzi et al., 2007). These minerals appear abundantly at the surface of the Earth as natural nanoscale particles with layered structures and interlayer spaces, which have potential to play a significant role as biomaterials. Structurally, the layers, with thickness around one nanometre, consist in most cases of one or two tetrahedral silicate sheets and one octahedral metal oxide/hydroxide sheet. Many clay minerals form sheet-like particles or platelets, for example, kaolinite (a 1:1-type clay mineral) formed by one tetrahedral sheet and one octahedral sheet, or talc, vermiculite, montmorillonite or saponite and others (2:1-type clay minerals), which are composed of an octahedral sheet sandwiched between two tetrahedral silicate sheets. Chemically, clay minerals are hydrous aluminium or magnesium phyllosilicates, usually with variable amounts of iron, magnesium, alkali metals, alkaline earths, and other cations present either in the interlayer space or in the lattice framework by isomorphous substitution. Uses of clay minerals in healthcare include traditional applications but also, as a result of the nanometre-scale layering, interlayer spacing and strong electrostatic interactions, the possibility of developing functional materials for such advanced technologies as nanotechnology and, in particular, biomaterials. Clay minerals and zeolites have been proposed as carriers of drugs, genes and proteins (López-Galindo and Viseras, 2004). The investigation of geomaterials–drug interaction and release mechanisms is an essential contribution for the formulation of geomaterial-based drug delivery systems. The possibilities of such systems depend on the amount of drug retained by the geomaterial as well as on the release kinetics and the total amount released in regard of the therapeutic regime.

Some polymorphic forms of silicates resulted in structures with particular interest. As for example, natural mineral nanotubes comprise hollow tubular minerals with nanoscale diameters.

Modification, functionalization and polymer conjugation of geomaterials.

Sometimes, properties of natural geomaterials cannot achieve the desired objectives, requiring their modification or functionalization as well as the incorporation into polymeric matrices to obtain nanocomposites with improved properties compared to the individual components (Viseras et al., 2008). All these improvements are notably prompting the availability of optimized and well characterized geomaterial-derivatives, suitable for a wide range of biomedical applications.

Concluding remarks

The therapeutic advantages of geomaterials in the design and development of technologically advanced drug products make this field of investigation a high-priority development area in which the development of new applications will require the close collaboration of pharmaceutical technologist with geologist, and chemists.

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University Institute for positron emission tomography in Skopje - unique facility for the new challenges in the regional health care system

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Background

The objective of our new facility of the University Institute of Positron-Emission Tomography are the production of PET radiopharmaceuticals for clinical service of in-house patients, commercial distribution of PET radiopharmaceuticals and precursors and development of new PET radiopharmaceuticals for diagnostic and therapeutical purpose. The factors foremost in the planning and design phases were the current regulatory climate for PET radiopharmaceutical production, radiation safety issues, and effective production flow. A medium-energy cyclotron (16.5 MeV) was installed in a bunker with the high-proton energy to offer a higher product radioactivity. This new state-of-the-art Positron Emission Tomography (PET) Institute and with included research capabilities is dedicated to providing the highest quality of nuclear imaging research.

Positron emission tomography in the last decade is one of the most promising methods of detecting oncological, cardiological, neurological diseases and enters slowly in the other fields showing hopeful results. The introduction of the new radiopharmaceutical for diagnosis and therapy in the clinical practice including the clinical trials are possible only with the appropriate production site according all cGMP requirements.

The new University Institute for Positron Emission Tomography is in the final official establishment as a unique facility in the country and in the Balkan Region. The new facility is result of the Government investment and joint project with International Atomic Energy Agency (IAEA).

Methodology

Establishing a PET institution is a large scale process that requires careful planning, inputs from multiple stakeholders, the support and approval of the authorities, secure funding, and a detailed implementation strategy. The need for a carefully planned strategy is even more essential in the conditions prevailing in a developing country, where the introduction of PET may be impeded by a scarcity of financial resources and, in many cases, an inadequate understanding of the potential roles and contributions PET imaging can play in a health care system.

Our new institution integrates Department for Production of Radiopharmaceuticals and Department of Molecular Imaging. They are connected in one unique system of Radiation protection, QA, BMS, Informatics and technical and administrative support of the network from the unified Health Care System in the country (IAEA, No.1, 2009).

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Results

The facility include cyclotron (GE, PET trace 860) for production of ultra-short-lived isotopes (F-18, C-11, N-13 and optional for solid targets that is planned already during the construction). Adjacent to the cyclotron is an area that houses the support equipment and a large dedicated workshop to support machine maintenance and target development. The Radiopharmacy production site contains two clean room with controlled-air environment class 10,000 (M5.5) and access via an interlocking entry change area. One completely dedicated for FDG production and second for the production of other F18, C-11, N-13, Ga-68 radiopharmaceuticals and Cu-64. The third production laboratory is dedicated for small scale production of diagnostic and therapeutical radiopharmaceuticals for clinical trials and investigation (White S, 2016). A fully shielded hot cells (class 100 [M3.5]) is located in all clean rooms. The PET radiopharmaceuticals are delivered via shielded tubing between the synthesizers and hot cells. Inside the hot cells, there is an automated device for dispensing the PET radiopharmaceuticals into either a bulk-activity vial or a unit-dose syringe (IAEA 2008). The dispensed PET radiopharmaceutical then passes through a hatch to a dedicated area where it is packaged for in-house use or commercial distribution. Unit doses for in-house patients are transported via elevator to the PET imaging area away. There is extensive radiation area monitoring throughout the facility that continuously measures radiation levels (IAEA No.1, No.58, 2009).

The integrated parts are two QC laboratories well equipped and one research laboratory for preclinical investigation including toxicological studies of new radiopharmaceuticals and imaging of small animal models.

The Department of Molecular Imaging is located in the second floor with two PET/CT cameras dedicated for clinical investigation and advanced biomedical imaging in human using extensive suite of state-of-the-art internally produced radiopharmaceuticals as imaging tools (IAEA 2010, Calabria and Schillaci, 2016).

This institution will be the full partner to the physicians not only in the country, but also in the region.

PET in that condition may serve as a magnet for recruitment in many areas and promote national and international interdisciplinary cooperation, to provide university educational opportunities for master, doctoral and postdoctoral studies, specialties in Nuclear Medicine, Radiopharmacy and Medical Physics with distinctive strength in education and research and an entrepreneurial dimension.

Conclusion

The University Institute for Positron Emission Tomography in Skopje will move a nationally and internationally recognized unit with true integration of research and service to the high level. The heath care for patients will be improved in many aspects and will help keeping the PET at the forefront of imaging procedures. Our Institute will serve as a model for future and more widespread clinical use of PET radiopharmaceuticals and provide proficiencies how PET technology may facilitate research and development opportunities.

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Design and evaluation of differently produced glyceride based mini-matrices as extended release systems for highly soluble model drug

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Introduction

Mini-tablets (d≤3mm or 5 mm) are emerging as promising type of drug delivery platoform suitable in the production of both single- or multiple-unit systems. Mini-tablets proved to be suitable dosage forms for targeted patient groups such as pediatrics, geriatrics and hospitalized patients since they may offer ease in swallowing (alone or mixed with soft food) and flexible dosing regarding age/weight/condition. When formulated as multiple unit systems, mini-tablets offer broad GIT distribution, less significant ''all or nothing'' effect and combining different release kinetics/compounds in one system (Aleksovski et al., 2015).

Hot-melt extrusion (HME) using circular dies and subsequent extrudate cut is emerging as promising technology in the production of mini-matrices aimed to deliver API by modified fashion. HME is solvent free process based on mixing, kneading, melting and transporting powdered materials through a pre-heated barrel equipped with one or two rotating screws up to an end plate die which determines final size and shape of the extrudate (Repka et al., 2012).

The aim of this research was to develop and evaluate mini-matrices based on either glyceryl behenate (GB) or glyceryl palmitostearate (GPS) for extended drug release of highly soluble model drug metoprolol tartrate. Mini-matrices were produced by two different techniques: hot-melt extrusion (mini-extrudates, EX) and compression of mini-tablets from untreated powder mixtures (direct compressed mini-tablets, DCMT) or granules obtained by milling extrudates (granule based mini-tablets, GMT).

Materials and methods

The following materials were used: metoprolol tartrate (MPT, EsteveQuimica) (20-40%), glyceryl behenate(GB, Compritol 888 ATO, Gattefosse) or glyceryl palmitostearate(GPS, Precirol ATO 5, Gattefosse) (80-60%), colloidal Si dioxide (0.5% in extrudates and tablets) and magnesium stearate (1% in tablets). The last two were replacing suitable amount of the glyceride.

Production methods: HME was performedon a co-rotating twin screw extruder with pre-heated barrel segments (entry to die T°C, GB EX -77/75/75/75/72/66 and GPS EX - 57/57/57/55/53/50). Obtained strains (d=3mm) were manually cut into mini-extrudates of 3 or 5 mm height. Mini-tablets (30 mg, d = 4mm) were produced on a single punch tablet press by compressing untreated powder mixtures (DCMT) or milled extrudates (GMT) 3kN compression force. When investigating influence of granule size on drug release, GMT samples were prepared by granules in the range of 0.150 mm ≤ d ≤ 0.250 mm (GMT S) or granules ranging 0.500 mm ≤ d ≤ 0.750 mm (GMT L).

Evaluation methods: Drug release studies (USP I) were carried out in H_2O, 0.1 M HCl, phosphate buffer pH 6.8, FESSIF with addition of pancreatin and $CaCl_2$ and blank FESSIF (without pancreatin, CaCl2, lechitin, and Na taurocholate). Calculation of similarity factor *f2* was performed. Solid state studies were performed using DSC and X-ray diffracttometry. Drug- glyceride interactions were evaluated by ATR-FTIR, while drug distribution by Raman mapping. Matrix porosity was determined by microcomputed tomography (μCT). Stability testing was performed at room and accelerated conditions in vapor protective bags.

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Results and discussion

HME and granule compression proved to be robust and reliable techniques when producing mini-matrices for extended drug release in all drug to glyceride ratios. Direct compression was seen as unsuitable technique due to poor flow properties of powders. Drug release from both freshly produced GB and GPS EX, GMT and DCMT was affected by the MPT loading.Increasing the loading (from 20% up to 40%) led into faster drug delivery, supposedly due to increase in system's hydrophilicity and formation of more open pore structure and thus more rapid MPT leaching in the medium. Formulations produced by melting technology (EX and GMT) provided increased embedment of the drug in the hydrophobic carrier and thus provided slower extended release pattern compared to formulations made by direct compression (DCMT). EX and GMT formulations based on 20% MPT were chosen for further studies due to most suitable prolonged drug release outcome over 24 hours. DCMT was not selected as further platform due to abovementioned problems with poor flow properties and the low extent of drug release retardation. When comparing the delivery of 20% MPT form either GB or GPS units, GBEX and GB GMT matrices gave similar delivery patterns between themselves (square root of time pattern, $f2$=72) while in case of GPS EX and GPS GMT release outcomes were versatile with EX sample showing specific sigmoidal release fashion and GMT square root of time release profile ($f2$=28). The sigmoidal outcome could be related to the appearance of ''wall depletion'' effect, where due to shearing appearance at the inner surface of the extruder MPT is emerging towards the extrudateinterior leaving very thin layer containing mainly glyceride which behaves as a diffusion barrier and thus limits drug release just from the cut sides of the matrix surface. During dissolution GPS EX cracks (after 8 h) which provides faster delivery rate and thus appearance of sigmoidal release pattern. GB EX and GB GMT showed higher porosity and thus faster drug release compared to the same units based on GPS. Porosity of GB EX is probably high enough to overcome the ''wall depletion'' effect. GB EX has more porous and less dense surface appearance compared to GPS EX. Milling the EX breaks the thin glyceride layer and thus disable its action as diffusion barrier and results in faster release profile of GPS GMT sample. Solid state properties (thermal and diffractional) of freshly produced matrices were slightly different than the ones of physical mixtures and also pointed partial solubilisation of MPT in the molten glyceride. Raman mapping and ATR-FTIR measurements indicated uniform drug distribution through either GB or GPS matrices and no significant interactions between the drug and used glycerides, respectively. Increasing the size of the EX from 3mm to 5mm in both GB and GPS samples led into decreased delivery rate due to increased diffusion route and decreased specific surface area of the units. Increasing the size of the granules in GB GMT led to faster release pattern of GB GMT L compared to GB GMT S due to higher porosity and larger pores of first ones compared to last ones. GPS GMT showed in general lower porosity compared to GB GMT and thus probably better compactibility of GPS granulate, which minimized the impact of the particle size on the drug delivery profile. GB units were insignificantly affected by the pH of the dissolution medium and presence of biorelevant compounds while GPS samples demonstrated faster delivery rate in phosphate buffer pH 6.8 compared to 0.1 M HCl and in FESSIF compared to blank FESSIF. Glycerides having higher acid number (GPS ≤ 6mg KOH/g vs GB ≤ 4mg KOH/g) and shorter fatty acid chains (GPS: C16 and C18 vs GB: C22) tend to exhibit increased ionization at phosphate buffer pH (decreasing hydrophobicity) and are more prone to lypolisis respectively, therefore giving slightly faster drug release outcome. Storing of GB units and GPS units at increased temperature for two months provoked changes in glyceride's solid state properties (GB – thermal changes: increased enthalpy and melting point maximum; GPS –thermal changes: increased enthalpy and peak maximum and change in the peak appearance; x-ray pattern changes), which was related to the alterations seen in the unit's drug release outcome after storage. GB units gave slower drug release probably due to blooming effect, while GPS units gave faster release probably due to increase in unit's porosity after storage. Storing units at room conditions affected only GPS samples,leading to faster drug release compared to freshly prepared units.Drug release changes were again connected to changes in the solid state properties of the glyceride. After thermal processing glycerides appear in a layered forms with lower crystallinityand are with time and other factors (increased temperature) transformed to a more stable state. Glycerides composed of longer fatty acid chains such as GB tend to change their crystalline form slower and at more extreme environment conditions, while glycerides based on shorter fatty acid chains such as GPS are prone to faster crystal alterations already at less extreme conditions.

Conclusion

Obtained results outline GB as more reliable matrix former for developing extended release mini-matrices compared to GPS. HME appeared as robust and reproducible process for mini-matrix production. However, understanding of material features and, HME equipment, process optimization and coupling with suitable down-stream processing are prerequisites for successful introduction of this technique as a viable alternative to the compression of mini-tablets.

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Formulation of chronotherapeutic delivery systems for delayed release of verapamil hydrochloride using polyethylene oxide polymers

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Introduction

Chronotherapy refers to a treatment method in which in vivo drug availability is timed to match rhythms of disease in order to optimize therapeutic outcomes and minimize side effects (Sajan et al, 2009). The pulsatile drug delivery system is intended to deliver a rapid drug release after a predetermined lag time (Kalantzi et al., 2009). Simple and inexpensive production methods, such as compression-coating of tablets, might be effective for these formulations to gain widespread use. Compression-coated tablets are composed of an inner core (immediate release tablet) which is embedded in an outer coating shell containing either a hydrophilic, hydrophobic or a mixture of both polymers, that dissolves or disintegrates slowly to produce the lag time. The outer layer surrounds the inner core, therefore selection of the outer layer materials has a significant impact on the performance of the tablet, including the mechanical strength, drug release characteristics, and tablet stability (Lin and Kawashima, 2012).

The possibility of formulating compression-coated tablets of verapamil hydrochloride was examined using polyethylene oxide (PEO) as polymer in the outer layer. The influence of different types and concentrations of polyethylene oxides (PEOs) on the drug release rate was investigated. Verapamil hydrochloride was selected as the model drug since the symptoms of hypertension are more prevalent during the early hours of the morning and development of chronotherapeutic formulation could be useful to fulfill the needs of drug delivery at the required time.

Materials and Methods

Materials

Different grades of polyethylene oxide polymers – Polyox® WSR 1105 (Mr ~ 0.9 x 106) and Polyox® WSR 301 (Mr ~ 7 x 106) were kindly provided by the manufacturer (Dow Chemical Company, USA). Verapamil Hydrochloride (Ph. Eur. 8.0), direct compression excipient based on coprocessed lactose and polyvinylpyrrolidone (Ludipress®, BASF AG, Germany), and anhydrous colloidal silicon dioxide (AEROSIL® 200 Pharma, EVONIK Industries AG, Germany) were used for the tablets preparation.

Methods

Preparation of tablet cores and compression-coated tablets

The inner cores of the compression-coated tablets were prepared by direct compression on an eccentric tableting machine (Korsch EK-0, Germany) using 9 mm concave punches. Tableting blend consisted of verapamil hydrochloride (30% w/w), anhydrous colloidal silicon dioxide (1% w/w) and Ludipress® as a diluent.

Compression-coated tablets consisted of the core tablet and the PEO polymer (Polyox® WSR 1105 or Polyox® WSR 301) as the outer coating agent. F 1 and F 2 batches were prepared using Polyox® WSR 1105 with core: polymer at the following ratios 1:1 and 1:2, respectively. Batch-

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es P 1 and P 2 were prepared using Polyox® WSR 301 with core: polymer at the same ratios 1:1 and 1:2, respectively. Firstly, half of the amount of the polymer (outer layer) was placed in the die to make a powder bed. Then, the core tablet was carefully placed in the centre of the die and the remaining half of the polymer was filled into the die. The contents were then compressed using the eccentric tableting machine (Korsch EK-0, Germany) to form a flat-faced tablet with a diameter of 12 mm.

In vitro drug release studies

The prepared core and compression coated tablets were subjected to in-vitro drug release studies in suitable dissolution media to assess their ability to provide the desired release. The *in-vitro* drug release study of the core tablets was performed in the rotating paddle apparatus (Erweka DT600, Germany). The dissolution medium was 900 mL of phosphate buffer (pH=6.8) maintained at temperature 37 ± 0.5 °C, and the rotating paddle speed was 50 rpm. The in-vitro drug release study of the compression coated tablets was performed in the reciprocating cylinder apparatus (VanKel's Bio-Dis, USA). The dissolution medium was 250 mL of 0.1 N HCl for the first 2 h followed by 6.8 pH phosphate buffer for the remaining period. Temperature of the medium was maintained at 37 ± 0.5 °C, and the dpm rate was set at 10 dpm.

In all experiments, an aliquot of 5 mL sample was withdrawn at the predetermined time intervals and replaced with an equal volume of drug-free dissolution fluid in order to maintain the sink conditions. The samples were filtered and analyzed spectrophotometrically at 273 nm by using UV/VIS spectrophotometer Evolution 300 (Thermo Fisher Scientific, Cambridge, UK). The amounts released were expressed as a percentage of the drug content.

Results and discussion

The prepared core tablets showed immediate release with more than 80% of the drug released in less than 15 minutes. With compressed-coated tablets, different release patterns were obtained using either different polymer type or polymer concentration. It was observed that the lag time increased as the concentration of the PEO in the outer coat increased. For instance, formulation F 1 had a lag time of 2 hrs before burst release. On the other hand, F 2 had lag time of 4 hrs. For batches P 1 and P 2, the results indicated that that the burst release of the drug occurred at 3 and 6 hrs, respectively. This also confirms that the lag time increased as the concentration of the PEO in the outer coat increased, but this increase were different with different types of polymers. An increased molecular weight leads to increase in polymer chain length and greater degree of chain entanglement, therefore stronger gel layers are formed in contact with water (Colorcon, 2009). Stronger gel layer with the greater viscosity decreases drug diffusion rate and water diffusion within the core tablet which consequently delays the drug release. Lower molecular weight PEOs form weaker gel layer, which is more susceptible to erosion. There is no general rule for selection of the appropriate polymer concentration that should be optimized taking into account the formulation composition, polymer molecular weight and drug solubility.

Conclusion

In the present investigation, compression-coated tablets with Polyox® WSR 1105 and Polyox® WSR 301 in outer layer showed delayed-release, and drug release from formulated tablets showed the dependence on both polymer concentration and its molecular weight. Further optimization of the Polyox® WSR 1105 and Polyox® WSR 301 coated tablets could produce the desired predetermined lag time.

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The role of cocrystallization screening for the assessment of structure-activity relationship in drug development

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Introduction

The selection of the crystalline phases in a form of molecular co-crystals has become scientific challenge at the early stage of drug development of pharmaceutical formulations and in the late stage of synthesis and isolation of active pharmaceutical ingredients (APIs) in desirable defined crystalline forms. Optimal crystal form of API interactively interrelates and impacts its aqueous solubility and dissolution rate that are benchmark for drug delivery and absorption determining the extent of its bioavailability and pharmacokinetics profile. Determining the crystal structure and revealing the crystal packing forces and geometry of the API has impact its physicochemical properties. This approach is the criteria for assessment of the performance of the API. The range of crystal forms in which molecular co-crystals of APIs may exist is advantageous comparing to their polymorphs, salts, solvates and hydrates due to the vast number of potential co-formers which extend the limited counterions for salt formation implying the existence of more complex intermolecular interactions based on different H-bonding patterns with API that lead to conformational changes and flexibility for crystal packing in process of co-crystallization.

Co-crystallization became well known bottom-up approach starting from intermolecular interactions among either selected neutral, ionic or zwitterionic molecules to design and control the properties of the multicomponent crystals (Braga, 2004). In the scope of interest for drug design and formulation, Good et al. (2009) and Cheney et al. (2011) emphasized that the main advantage for designing pharmaceutical co-crystals (PCCs) is, through their modulating properties, to improve the performance of the native APIs such are: biopharmaceutical profile (solubility and dissolution rate), thermodynamical stability (phase transition of polymorphs, solvate/ hydrate formation, decomposition) or bulk powder processability (flowability, compressibility, particle size and shape control). Childs et al. (2007) has pointed out the necessity co-crystals (CCs) semantically to be classify based on accomplishments in research of supramolecular chemistry. This approach enlightens the complex reality of multi-component systems and the wide scope associated between salts and co-crystals, and their differences based on the location of the transferred proton within the salt - co-crystal continuum.

Biguanide drugs are well known and wide used oral antidiabetic drugs for oral therapy of diabetes type-2 that directly improve insulin action. Recent studies in the research work carried out by Vujic et al. (2015) has pointed out that biguanides in combination with targeted inhibitors in order to obtain synergy in reduction cell viability, inhibited tumor growth in the mutated neuroblastoma rat sarcoma oncogene (NRAS) protein from melanoma cells. Hence, it is expected that combination of biguanides which affect activation of the AMP-activated protein kinases (AMPK) and the regulation of energy metabolism with outcome to cell's energy sparing, in combination with other anti-cancer drug-models would influence direct blocking cell's signaling and hinder the resistance.

Materials and methods

Co-crystallization screening reveals protocol was undertaken in order to grown single crystalline phases of PCCs composed of drug model metformin (MET), selected from biguanides class of drugs and coformers that belong to different pharmacotherapy and functional group classes, respectively.

Co-crystallization screening was carried out on applying slow-rate solvent evaporation method for growing sin-

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gle crystalline phases at room temperature with quality absolute structure to be determined. An equimolar quantity of MET and co-crystal partner was dissolved in the minimum quantity of ethanol and left for slow evaporation at room temperature. Colorless crystals were observed after a few days.

For four MET PCC models, the methods of preparation reproducible batches were optimized. The quality of batches was controlled by Powder x-ray diffractometer comparing the obtained experimental diffractograms with the same one that was theoretically generated from the single crystal for each of four PCC models.

Single-crystal diffraction data were collected on a Nonius Kappa diffractometer equipped with a CCD detector with graphite-monochromatized MoKα radiation (λ = 0.71069 Å). Intensities were corrected for Lorentz and polarization effects. The structures were solved by direct methods with the SIR97 suite of programs and refinement were performed on F2 by full-matrix least-squares methods with all non-hydrogen atoms anisotropic.

Flow-cytometry was applied for measuring viability of the two PCC models.

Results and discussion

Vujic et al. (2015) has carried out research for both pro-cancer and anti-cancer effects of biguanides on cancer cells, indicating existence of association of the antidiabetic therapy and reduced risk of cancer in diabetic patients. Because biguanide represents the π-conjugated system, MET can exist in three resonance-stabilized forms, i.e. as neutral molecule (MET), monoprotonated (MET+) or diprotonated (MET2+) cation, with dissociation constants in water in range from pKa1 ≈ 12.00 to pKa2 ≈ 2.00.

A search of the biguanide fragments in the structural literature, both in the CCDC (Cambridge Crystallography Database Center) database and in patents, shows that in crystals MET exists as monoprotonated (MET+) or deprotonated (MET2+) but never in its neutral form MET.

We have undertaken a systematic study of the crystal chemistry of MET with the aim of understanding its properties in the solid state and finding relationships with its biopharmaceutical profile. We have determined the structures of the 29 MET PCCs. Four of this MET PCC models are "drug-drug" type of co-crystals. The ligand used for co-crystallyzation was from the following classes: inorganic acids (nitric, phosphoric and carbonic acid); organic NH-type acids (saccharine and acesulfame); organic OH-type acids (squaric and picric acid); monocarboxylic acids (fumaric, acetic, trifluoroacetic, trichloroacetic, dichloroacetic, monochloroacetic, glycolic, salicylic, diclofenac) and dicarboxylic acids (oxalic, malonic, maleic, fumaric, succinic, adipic acid).

Conclusion

In the paper are presented structure analyses for "drug-drug" type of PCCs where both API and CF exhibit pharmacological effect. This approach of designing "drug-drug" type of PCC aligned to the strategy for drug repositioning, the idea for use of a drug for treating diseases other than the drug-specified. This concept was prompted in 2012 through the Discovering New Uses for Existing Molecules program, initiated by US's National Institute of Health (NIH).

The case study underlines the crystal growth and the method of preparation for "drug-drug" type of PCCs wherein two different APIs cocrystallized in single crystal cell, and that represent new paradigm for approaching in development of "fixed-doses" or "combo" pharmaceutical formulations. Preliminary results of the Structure-Activity Relationship study on the co-crystals composed of MET with dichloroacetic acid indicate dual and complementary anti-cancer activities of the two selected drug models for co-crystallization.

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Short communication

Recombinant monoclonal antibody rituximab – medical uses and structural characterization

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Introduction

This paper addresses current topics related to CD-20 depleting agent rituximab, which has been widely used as a targeted therapy in the fields of oncology and rheumatology. Apart from its structure and function, relevant issues regarding the analytical methodologies applied for the assessment of the quality of this complex drug will be highlighted as well.

Rituximab is the first approved targeted biologic therapy for the treatment of haematological malignancies and it took very short time from its first description until the approval and clinical application. In USA it was approved in 1997, whereas in EU in 1998. Approved clinical indications of rituximab include non-Hodgkin's lymphoma, chronic lymphocytic leukaemia, rheumatoid arthritis and granulomatosis with polyangiitis and microscopic polyangiitis (European Medicines Agency-EMA, 2016). Since the patents on MabThera and Rituxan expire in 2016 in USA and have already expired (2013) in EU, there are some biosimilars of rituximab in the late stages of clinical trials developed by companies worldwide, whereas 'non-originator biologicals' of rituximab are approved in some South American and Asia countries (GaBI, 2016). Pharmaceutical formulation (Rituxan or Mabthera) is administered intravenously and supplied at a rituximab concentration of 10 mg/ml in either 10 ml or 50 ml single-use vials.

Structure – activity relationship

Rituximab is genetically engineered chimeric monoclonal antibody (mAb) that is produced in mammalian cell culture using Chinese hamster ovary cells. This IgG1antibody contains murine light and heavy chain variable regions, and human gamma 1 heavy chain and kappa light chain constant regions. During the chimerization process, murine variable domain of heavy and light chain was cloned into the human IgG1 immunoglobulin framework. In the chimerised version only variable domains have murine origin (around 30%). The rest of molecule is human (around 70%). Rituximab binds specifically to the antigen CD20, which is found on the surface of normal and malignant B-lymphocytes (Reff et al., 1994), which in addition play a role in the pathogenesis of rheumatoid arthritis. The Fab domain of rituximab binds to the CD20 antigen on B lymphocytes, and the Fc domain recruits immune effector functions to mediate B-cell lysis through complement-dependent cytotoxicity (CDC), antibody-dependent cell mediated cytotoxicity (ADCC) and apoptosis.

As all biological drugs, rituximab has very complex nature, it contains 1328 amino acids with theoretical Mw of non- glycosylated form 144.54 kDa. It contains two identical heavy and two identical light chains linked via disulfide bridges. Each heavy chain contains 451 amino acids (49.2 kDa) and pI 8.67, whereas each light chain contains 213 amino acids (23.06.kDa) and pI 8.26. The apparent Mw of rituximab is higher than 144.54 kDa, due to the presence of N-linked oligosaccharides attached in the N-glycosilation site at conserved asparagines N-301. Variable regions of both chains comprise complementarity determining regions (CDR1-3), being responsible for antigen recognition and binding. On the other hand, human Fc γ1 (IgG1) fragment is responsible for ADCC and CDC. Heavy chains are linked *via* two interchain disulfide bonds at the flexible hinge region, while heavy and light chains are linked with a disulfide bridge.

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Structural characterization

As all biopharmaceuticals, especially mAbs, rituximab is extremely complex and heterogeneous drug. The main source of the antibody heterogeneity results from differences in protein glycosylation which is one of the most common post-translational modifications of proteins produced in eukaryotic cells although, primary structure variations, deamination, deamidation, lysine truncation, methionine oxidation, sulfation and phosphorilation can create additional IgG variants. The characterization and stability study of rituximab are extensive and require a battery of orthogonal techniques as amino acid analysis, amino-terminal sequence analysis, peptide mapping, and analysis of oligosaccharides, ion-exchange chromatography (IEC), cIEF, SDS-PAGE, and circular dichroism (CD), UV spectroscopy, size exclusion chromatography (SEC), cellular mediated cytotoxicity (CMC). The oligosaccharide structure was investigated by CZE and MALDI-TOF after isolation from HPAEC-PAD (European Medicines Agency-EMA, 2016). Two-dimensional gel electrophoresis (2-DE) complemented with MALDI TOF MS analysis was used for the characterization of identity, purity and structural integrity of rituximab. Experimental data revealed typical migration behavior of mAbs, resulting in poorly resolved spots with different isoelectric points and very small differences in Mw. Heavy chains migrated at about 50 kDa and light chains at about 23 kDa. After tryptic digestion of 2-DE separated proteins, peptide mass fingerprinting analysis was also used for the identification of rituximab heavy and light chains (Nebija et al., 2011). Glycosylation of IgG is critical for effector functions including complement fixation, Fc receptor binding on macrophages and ADCC. In addition clearance of IgG-antigen complexes from circulation is influenced by IgG glycosylation. It was shown that predominantly N-linked oligosaccharides present in rituximab belong to asialo, neutral complex biantennary oligosaccharide type different terminal galactose residues, whereas terminal sialylated oligosaccharides are present only in minor amounts (Kamoda et al., 2004).

Manufacturer of pharmaceutical product MabThera® as primary assay for characterization and lot release of N-linked glycans on glycoprotein drugs employed CE-LIF. A method for direct characterization of glycans using CE-LIF/MS has been reported, as well. Apart from major glycan components this method allowed accurate identification of minor glycans such are asialo- and afucosylated species. Since the fucosilation affects biological activity and sialylation affects the pharmacokinetics of glycoprotein drugs, the identification of these species is of particular significance for the characterization of rmAbs. Charged variants of rmAbs can be studied with different methods such as IEC, IEF, CZE. A simple and rapid method for determination of relative amounts of rituximab glycoforms differing in terminal galactose was reported. Most abundant ions corresponding to the glycoforms found on the rituximab heavy chain were monitored by mass selective detection in the selected-ion monitoring mode (Nebija et al., 2011).

Lot release testing of the pharmaceutical product Rituxan included physicochemical and biological methods, such as SDS-PAGE, peptide mapping, fragment IEC-HPLC, SECHPLC, glycan content, cIEF, CDC, UV-VIS. Validated human CDC assay was used for the determination of potency. Galactose content on the heavy chain oligosaccharide was found to be critical parameter for biological activity, therefore another release test for glycan content was developed (CE-LIF). cIEF was used to positively identify rituximab from other recombinant mAbs made by the manufacturer (US FDA CDER, 2016; Zhang et al., 2016).

Conclusion

As a conclusion, since rituximab belongs to rmAbs, class of drugs obtained by rDNA technology, biological processes are involved in its production. Therefore it demonstrates high degree of inherent heterogeneity and complexity and the combination of different techniques should be used for the extensive characterization of its quality attributes, including identity, structural integrity, purity and stability. This is of primary importance for the safety and efficacy of this class of drugs. In this regard, it was shown that gel electrophoresis and peptide mass fingerprinting analysis may represent an important strategy for the assessment of the quality of rituximab and other biopharmaceuticals.

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Short communication

Comparison of emollient efficacy - a single centre, randomised, double-blind, bi-lateral comparison of two emollients prescribed in the UK for the management of dry skin conditions such as atopic eczema

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Introduction

In the UK, the National Institute of Clinical Excellence (NICE) clinical guidance on atopic eczema management in children from birth to the age of 12 years establishes emollient therapy as the treatment modality that should underpin all else (NICE quick reference guide, 2007). The guidance recommends continuing emollient therapy even when the skin appears healthy. Healthcare professionals are advised to offer children with atopic eczema a regime of 'complete' emollient therapy involving a choice of non perfumed emollients to use every day, both as leave on moisturisers and as soap substitutes for routine washing and bathing.

Emollient formulations suitable for such use typically contain both oily occlusive substances, such as petrolatum, paraffin or mineral oil, which form a water impermeable film over the skin to decrease evaporation of physiological water from beneath, and humectant substances, such as glycerol and urea, which attract water to the skin (Cork, 2007; Loden, 2003; Rawlings et al., 2004; Watkins, 2008). In order to encourage treatment concordance, however, it is also crucially important that these products are formulated in such a way that their physical characteristics render them appealing for patients to use over large surface areas and for long periods (Cork et al., 2003).

Ideally, emollients should exert their skin softening and moisturising effects within the upper layers of the skin. Thick, greasy ointments undoubtedly exhibit good emollient characteristics, but are not very popular with patients because they are not well absorbed into the skin and leave an oily residue which can feel uncomfortable and has a tendency to soil clothing and bed linen (Cork, 2007; Sidbury and Poorsattar, 2006). More popular emollients are formulated as oil-in-water creams or lotions to make them 'feel' lightweight and to encourage better absorption into the skin (Ersser et al., 2007). However, these dosage forms are less effective than ointments owing to their lower oil content, resulting in reduced occlusive capabilities (Clark, 2004) Moreover, cream and lotion formulations exhibit poor substantivity and have a tendency to be easily rubbed off onto clothing, so they necessitate more frequent re-application than would be practicable for many patients. Emollient gels are alternative pharmaceutical presentations for atopic eczema sufferers and usually contain high concentrations of oily ingredients in semi-solid aqueous systems. To achieve maximum benefit, some of these gels, just like other emollients, need to be applied regularly and frequently.

Performance evaluation methods for emollients and moisturisers are mainly focused on sensory aspects, skin visual appearance, perceived efficacy, and measurements of skin barrier hydration and integrity (Rawlings et al., 2004). However, few studies have involved patients directly comparing the effectiveness of different emollients by using them concurrently or under conditions mimicking normal therapeutic use (Clark, 2004; Simpson, 2006).

Consequently, the aim of this study was therefore to compare the effects on skin hydration of two emollients prescribed in the UK, DELP gel and ZB cream, and using a dosage regimen consistent with most patients' practical circumstances which limits their use of emollients to twice daily only.

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Materials and methods

This was a single centre, randomised, double blind, concurrent bi-lateral (within-patient) comparison in 18 females with atopic eczema and dry skin of similar severity on their lower legs. Following 7 days' run-in with no use of emollients or moisturisers on the lower legs, DELP gel and ZB cream were each applied to one lower leg twice daily (approximately at 9am and 9pm) for 4 days and on the morning only on day 5. Washing of the lower legs was permitted only during the evening on days 2 and 4. The efficacy of both products was assessed by hydration measurements using a Corneometer CM825 probe (Courage-Khazaka electronic). The measurements were made on days 1 to 5 at approximately 9am immediately prior to the first daily application (the measurement on day 1 being baseline), and around 1pm and 5pm. The primary efficacy variable was the area under the curve (AUC) of the change from baseline corneometer readings over the 5 days.

Results and discussion

The two emollients showed very different effects on skin hydration. The AUC for DELP gel significantly outperformed ZB cream. For DELP gel, the skin hydration effect was substantial, long lasting and cumulative, with the readings each day generally increasing over the treatment period. Even the morning readings on days 3 and 5, following washing the previous evening, were significantly better than baseline. In contrast, for ZB cream, skin hydration was not significantly different from baseline at any time point.

Conclusion

This study, performed by subjects with atopic eczema and dry skin using a dosage regimen simulating normal/practical use, has demonstrated very significant performance differences between two marketed emollients. Whereas DELP gel achieved substantial, long lasting and cumulative skin hydration, ZB cream achieved no measurable improvement compared to before treatment. Healthcare professionals should be aware of this when prescribing these products.

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Implementation of mexametry in periorbital hyperpigmentations studies

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Introduction

Usually known as Periorbital Hyperpigmentation (POH), this deficiency seems to have in literature many other denominations, such as: Idiopatic Cutaneous Hyperchromia in the Orbital Region, Infraorbital dark circles, Cutaneous idiopatic hypercromia of the orbital region or Periorbital melanosis. The lack of a unique denomination should not be a serious problem, but behind these inconsistencies are usually some different approaches concerning the ethiopathogeny, as well as the targeted pathogenic treatment by default.

The literature data originating from European authors, as well as some articles of North Americans ones include the periorbital hypercolorations into the chronologic aging process, with implications resulted from vascular and sanguine deficiencies. In other words, it is considered that this deficiency is not necessarily originating from a genetic anomaly. This point of view is due to the fact that most of the subjects from this geographic area are of pale or fair skin phototype (I, II or II phototypes). According to Suppa et al. (2011) the determinants of periorbital pigmentations and of the skin ageing processes are: UV exposure, smoking, vascular problems. Lupo et al. (2011) consider the intrinsic aging role in the periorbital hyperpigmentations pathogeny and suggest a new topical treatment based on human growth factors, cytokines, caffeine and glycyrrhetinic acid.

On the other hand, literature data attributed to Asian or South American authors indicate that the primary cause of periorbital hypercolorations pathogeny should be rather melanic, constitutional, and they refer to as subocular melanosis. This is probably due to the dark skin phototype (IV, V and VI phototypes), specific in these geographic areas. Some authors point-out the post-inflammatory pigmentations' role onto color intensification of this pathogeny, while in other situations it is exclusively assigned to the melanic cause of this process (Kanika and Kassir, 2013). In the same time, in the case of subjects with dark skin phototype, it was stipulated that the main causes of periorbital hyperpigmentation could be melanic, without excluding the aging specific endogeneous processes as aggravating factors.

Corroborating these different approaches, one could consider that the main determinants of periorbital hyperpigmentations, for any skin phototype could be: the genetic melanic pigmentation, the post-inflammatory hyperpigmentation, a tegument periorbital atrophy due to chronoaging, the venous congestion, hemoglobin and oxygenated hemoglobin accumulations, hemosiderine deposit, as well as some other anatomic causes.

Materials and methods

The chemicals used in this study were of high purity grade. Acetyl tetrapeptide-5 (trade name EYESERYL®) was obtained as a gift from Lipotec S.A. (Isaac Peral, 17 Pol. Ind. Camí Ral; E-08850 Gavà Barcelona, SPAIN) as 0.1% aqueous solution. An o/w microemulsion was used as vehicle for the transport of acetyl tetrapeptide-5 through the skin. The main ingredients of the microemulsion were: deionised water, glycerol, liquid paraffin, vaseline, jellifying and lubricant mineral agents, emulsifying agent (Montanov L), preservative agents, and acetyl tetrapeptide-5. The rheological properties of the microemulsion were assessed using the Rheometer RC1 (Rheotec), and two type of probes: CC8 and C25-1.

The study was carried-on on 2 test groups of healthy women volunteers aged of 22 to 64 years. The first group applied on both (right and left) eyes hemispheres the o/w

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emulsion containing 0.1% peptidic complex, twice daily, during 60 days. The second group (control) applied the cosmetic emulsion containing 0.1% acetyl tetrapeptide-5 only on the right eye suborbital area, while the left eye was treated with the simple cosmetic formulation. The effects of the cosmetic emulsion were evaluated every 15 days, both by clinical and instrumental methods. The instrumental evaluation was performed by Mexametry, using the MX 18 Mexameter (Courage-Khazaka), which is able to measure two components, mainly responsible for the color of the skin: melanin and haemoglobin (erythema).

The aim of the present work was to study in vivo the efficiency of the acetyl tetrapeptide-5 in the treatment of periorbital hyperpigmentations and eye puffiness, using mexametry as one of the most recent non-invasive method of evaluation of the coloration's intensity of the sub-orbital area.

Results and discussion

In our study, the melanin parameter was excluded due to the fact that acetyl tetrapeptide-5 is not considered as a melanic depigmentant, lacking any tyrosine inhibitor in this composition. Considering that periorbital hyperpigmentations are dependent by individual metabolism, the relevant parameter used as a measure of suborbital coloration was the erythem one.

In the case of the control group, a significant reduction of the erythem values was obtained for the right eye, while for the left eye, the reduction of the erythem corresponding values was quite slight, or (in some cases) none, even at the end of the eight weeks of treatment. In the case of the study group which applied the o/w cosmetic emulsion containg the peptidic complex on both eyes hemispheres, significant reduction of the erythem values was recorded, in some cases even after 15 days of treatment. For this group, 25% of the volunteers showed a slight reduction, 30% a fairly good reduction, and 40% a good one.

Conclusion

In conclusion, mexametry was successfully used as instrumental non-invasive method for the investigation of periorbital hyperpigmentations as consequence of a specific cosmeceutic treatment. The obtained results showed that the cosmetic formulation containing different amounts of acetyl tetrapeptide-5 was well tolerated at the tegument level and visible results were obtained, even after only 15 days of treatment. Moreover, 95 % of the volunteers showed a significant reduction of the periorbital hyperpigmentations and eye puffiness after the treatment with the cosmetic preparation containing acetyl tetrapeptide-5.

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Short communication

A novel natural mixed emulsifier of alkyl polyglucoside type as liposome and skin-friendly cosmetic ingredient

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Introduction

Liposomes are phospholipid-based vesicular carriers that still attract great attention of formulators. However, the selection of an appropriate carrier for liposomes remains one of the most important issues. Although, gels are still considered as the most suitable bases for liposomes (Kulkarni, 2005), they are usually not appropriate, neither for the long-term care nor for all skin types. Therefore, emulsion systems are believed to be more convenient carriers. On the other hand, formulation of an emulsion with liposomes entails another problem: selection of suitable emulsifier(s) necessary for system stabilization, considering the fact that emulsifier may interfere with the mechanical stability of vesicles and may enhance propensity for fusion or lead to their solubilization (Kulkarni, 2005). An interesting and highly promising group of novel emulsifiers which could be used for development of emulsion carriers for liposomes are alkyl polyglucosides (APGs). Due to their favorable toxicological and dermatological properties they are considered as environment- and skin-friendly (Lukic et al., 2013).

Therefore, our aim was to investigated whether a novel APG emulsifier, hydroxystearyl alcohol & hydroxystearyl glucoside, could be considered as an appropriate stabilizer for emulsions containing liposomes. To accomplish this, a model emulsion carrier with liposomes was formulated and characterized first. Additionally, the in vivo irritation potential of the developed formulation was assessed, as a certain aspect of safety of the used emulsifier.

Materials and methods

Hydroxystearyl alcohol & hydroxystearyl glucoside was used for samples' preparation with ARSC-liposomes-stem cells of alpine rose leaves in liposomes as an active.

Two oil-in-water (o/w) creams (placebo Fp and active cream F1a containing 0.4% (w/w) of ARSC-liposomes) were developed and characterized by means of: polarization microscopy, rheology, differential scanning calorimetry and thermogravimetric analysis as described (Lukic et al., 2013). Afterwards, an in vivo study with investigated creams and commercially available cream containing the same active was conducted. Skin hydration (EC), transepidermal water loss (TEWL), and erythema index (EI) were measured in accordance with the Declaration of Helsinki and relevant guidelines on 16 healthy volunteers.

Results and discussion

Micrographs taken after 7 days revealed randomly distributed distorted Maltese crosses and birefringence at the oil droplets border ("onion rings") within both samples (Fp and F1a), indicating lyotropic interaction of lamellar type (Lukic et al., 2013) and remained relatively unaltered after 30 days. However, obtained micrographs showed the noticeable difference in the colloidal structure between the placebo Fp and the active cream F1a. Considering that liposomes are also visible under polarization microscopy, it seems that liposome-similar structures could be observed near the lamellar phase gel network i.e. close to the "onion rings" in the continual aqueous phase. Intact vesicles were apparently immobilized within the network of lamellar phase of the system and as such are mechanically stabilized, hence their interaction and fusion are limited.

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Continual rheology has shown that both samples exhibited "shear-thinning" (pseudoplastic) flow behavior with pronounced thixotropy. The obtained flow curves and calculated hysteresis area values showed that the addition of the 0.4% (w/w) of ARSC-liposomes has led to a slight increase in viscosity and thickening of the placebo cream, but rheological behavior of both creams stayed rather similar. Oscillation frequency sweep test has shown prevalence of the elastic over the viscous component as the one of the general characteristics of lamellar phase (Lukic et al., 2013) for both samples. Active sample (F1a) had higher elastic-storage modulus (G') and viscous-loss modulus (G'') compared to the placebo (Fp) which is in accordance with the result of continual rheological measurements. Increments in all the assessed rheological parameters upon the addition of ARSC-liposomes, could be due to lamellar phase promotion by the cosmetic active and the change in orientation of the lamellae under shear stress or due to the presence of intact liposomes themselves.

Thermal behavior of the investigated samples (Fp, F1a) was similar in terms of the shape of the obtained profiles with one marked peak. The addition of the liposome-encapsulated active (sample F1a) induces a subtle shift of the curve towards higher peak temperature values. Although such results may imply the promotion of the lamellar phase (Lukic et al., 2013), considering the polarization micrographs and the rheology results, it is more likely that the detected thermal behavior is due to ARSC-liposomes themselves.

Regarding TGA results, upon the addition of the active, amounts of the lost water decreased in the first temperature range (25-50 °C, corresponds to the free water in the system) and increased in the third temperature range (70-110 °C, corresponds to interlamellar water) (Lukic et al., 2013). After the addition of ARSC-liposomes there was no change in the percentage of lost water in the second temperature range (50-70 °C, corresponds to the water bonded within the lipophilic gel phase). Based on all results, we assume that vesicles packed near the lamellar crystalline phase around the oil droplets and their inherent water-binding capacity jointly led to the observed transport of the water within the system.

In order to assess the in vivo irritation potential of samples (Fp, F1a and Fc) and the investigated emulsifier, EI was monitored. Additionally, the potential skin barrier impairment was assessed via TEWL and EC. After the 24-hour occlusion, EI was not significantly changed compared to the baseline values for any tested cream. TEWL was significantly decreased for the creams Fp and F1a compared to the baseline. Considering that TEWL was not significantly changed for the commercial cream Fc (the same active, but different carrier), it could be speculated that these results can be attributed to the carrier itself (system with the liquid crystalline structure) and the used emulsifier. Assumption is in accordance with the reported results concerning skin mildness of this type of emulsifiers (Lukic et al., 2013). Regarding EC, it was significantly increased compared to the baseline values in all the treated sites, apart from the placebo sample Fp where merely a trend of increase was observed. Since there was no significant change in the EC for the untreated control under occlusion, the obtained results cannot be attributed to the occlusion. The absence of erythema and/or any impairment of the skin barrier function in a 24-h occlusion study may preliminarily imply a satisfying safety profile of the creams and the used APG emulsifier.

Conclusion

The study confirmed that a novel alkyl polyglucoside emulsifier, hydroxystearyl alcohol & hydroxystearyl glucoside, could be classified as liposome-friendly. It was shown that in the system stabilized with lamellar phases, liposomes are apparently immobilized within its network and as such are mechanically stabilized. Additionally, due to the lack of skin irritation and skin barrier impairment during the application on healthy skin, it could be said that the investigated emulsifier is skin-friendly and may be safely applied as stabilizer for cosmetic or prospective pharmaceutical emulsion carrier for liposomes.

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Short communication

Development of an improved method for the *in vitro* determination of the Sun Protection Factor (SPF) for sunscreens

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Introduction

Sunscreen products are cosmetics according to Council Directive 76/768/EC (CD 76/768/EC, 1976). The efficacy and moreover the safety of sunscreen products, as well as the basis upon which they are claimed are part of extensive research because of the importance over the general human health. For the regulation of the sunscreen products referral to the EC 1223/2009 of the European Parliament (EC1223/2009, 2009) and to the Recommendation EC 2006/647 (2006) on the efficacy and claims of sunscreen products was done.

Based on this legislation it is necessary to validate the photo protection separately in the range of UVA and UVB, with *in vitro* and *in vivo* methods, with preference for the latter. The UVA range (320 - 400 nm) *in vivo* tests have been replaced with the *in vitro* tests, because of the consideration of the good correlation found, harmonized and standardized in the ISO 24443 (2012). For the UVB range (290 - 320 nm) the use of the method *in vivo* is still a necessity, standardized by Standard ISO 24444 (2010), due to problems related to lack of correlation between the *in vitro* and *in vivo* data. The Sun Protection Factor (SPF) continues to be widely used for describing the real performance of the product as well as its safety, and it is sometimes misunderstood, as the unique indicator for the efficacy and safety. Besides the numerical value of the SPF other properties and factors have to be taken into account by the consumers. During the last decade the sunscreen formulations are enriched with many other ingredients such as boosters, antioxidants, immune-modulators. Also into many already marketed products for skin care and make up sunscreen filters are added.

Accordingly, it is more appropriate to describe performance as a whole formula protection factor: Formulation Efficiency Factor (FEF) calculated as SPF/actives % x 100 (O'Lenick and Lott, 2011). FEF as a new parameter in the sunscreen research relates the amount of active sunscreens in a formulation to the overall SPF of the finished product. A formulation chemist can quickly and accurately apply the method to access the efficiency of a certain formulation. Sunscreens can be classified into three major categories based on the formulation active ingredients: organic, inorganic/organic and inorganic. In this complex pattern and to properly address our work toward the development of safe and effective solar products, we investigated the factors that influence the in vitro SPF determination. Having increasing problems in the determination of in vitro SPF values predictive of the in vivo assessment. With this work, we intended to evaluate, at first repeatability of the measure and, secondly, the accuracy of the same.

Test variables of the *in vitro* SPF determination, such as substrate surface temperature, substrate choice and pressure of sunscreen spreading have been examined (Miksa et al., 2013).

The purpose of this study was to investigate the impact of the type of product, way of application and method used to determine *in vitro* SPF.

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Materials and methods

Sun Protection Factor (SPF) measurement and in vivo *correlation*

SPF *in vitro* assessment was carried out on 80 random commercially available products, beside the 3 standard products prepared by us as reference formulating SPF from low and medium to high. Two different protocols for *in vitro* determination were used: the Diffey-Robson method (method A) and the ISO-24443 (method B), using tape and PMMA plates respectively with two different pressured applied (100 and 200 g) for spreadability. *In vivo* tests of selected products were also obtained for comparison using a solar simulator SPF Ultraviolet Solar Simulator 600-150 W/300 W Multiport that provides ultraviolet radiation in the region between 290 and 400 nm from 6 independent outputs. The results were collected on 10 volunteers male and female, belonging to the phototype I, II and III, aged between 20 and 35 years.

Different PMMA's and surgical tape Transpore TM, quantity of product, spectrophotometers (JascoV530PC and Shimadzu UV-2600) and method of application (manual and mechanical) were examined. For the ISO-24443 (method B), the tested product was applied to a new PMMA plate of 5 cm x 5 cm area, and 5 µm of roughness (Schonberg GmbH). The application rate of 0.0128 g/cm^2 ± 0.0003 g was controlled by mass. The application dose was determined by measuring the plates before and after the spreading operation. The application of the product has been realized according to the ISO 24443 (2012) guidelines by spotting, with a pipette, the product on several points all over the plate surface and then distributed by a fingertip, pre-conditioned with the testing sample, for 30 second, with light circular movements; the plate is then positioned on a scale where the spreading phase is carry out performing a pattern of movements in horizontal in vertical direction, checking the pressure applied in all the moments. For all the products the spreading pressure is first of 100 g and in a second analyze of 200 g. Before the measure the sample lies for a minimum of 30 minutes in a dark place. The spectrophotometer used in this method is a Shimadzu UV 2600 provided of integrating sphere ISR 2600 60 mm, and coupled with a SPF determination software: for each sample the transmittance is measured from 290 to 400 nm. Every product has been measured 3 times, i.e. three different product applications on three different plates. Each sample has been tested in six different areas at least; therefore the results presented come from an average of 15 set of data.

Results and discussion

The *in vivo* test is only a recommendation (EC 2006/647, 2006) and in fact many companies, limited by the cost of the in vivo procedure, are often relying on solely the results from the *in vitro* or even on simulations from calculating software, based on the concentration of filters used. In our investigation, the limited reliability of *in vitro* method was confirmed on complex formulation. The calculated SPF data indicate that the spreading method developed in our laboratory is reproducible for all the product test e with Cov% values less than 5%. Most accurate results were obtained with the B method that, in terms of internal repeatability and of acceptance parameters, provided some correspondence with values obtained *in vivo*, in particular using PMMA plates standardized as the roughness, pressure, amount of product and way of application. In conclusion, our method B gave statistically sound results and the better correlation between the *in vitro* and *in vivo* data, these data although preliminary, encourages us to further extend the study to a larger number of samples in order to better understand if any affordable method can be drawn on the basis of these premises.

Conclusion

Our study indicates that the variability of response is largely related with the standardization and thus harmonization of the procedure between different labs and thus we suggest joint cooperative efforts toward a possible ISO definition of an SPF-Vitro protocol.

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Short communication

Emollient gels: characterisation of physical structure and behaviour in the presence of salts

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Introduction

Emollients are therapeutic moisturisers that are usually available as leave-on formulations of various types i.e. creams, ointments, lotions and gels (Ersser et al., 2007). These products are used for the treatment of dry skin conditions such as eczema and psoriasis (Voegeli, 2011). Due to the wide range of products available on the market it can be difficult to recommend the most effective and appropriate emollients for the patient. As such, prescribers tend to recommend leave-on topical emollient products based primarily on patient preference and cost (Moncrieff et al., 2013). However, to achieve maximum benefit the emollient product must be both clinically effective as well as cosmetically appealing (Dederen et al., 2013). It is also recognised that emollient preparations are not the same even if claimed to be equivalent to other products, hence prescribers are encouraged to avoid false economy in their prescribing practices (Moncrieff et al., 2013). It is therefore critical for manufacturers to develop innovative products with optimised clinical performance and appealing sensory characteristics (Herman, 2007). To achieve this, application of simple and cost-effective analytical methodologies/approaches that can screen and compare such characteristics would be advantageous (Inoue et al., 2013; Stojiljković et al., 2013).

Emollient gels are considered one of the most cosmetically acceptable leave-on topical formulations, because of their high water content and non-greasy feel (Ersser et al., 2007). These emollient formulations are oil-in-water (O/W) dispersions emulsified using, in general, carbomer gelling agents. The gelling agent(s) act as a physical stabilizer of the dispersed oil droplets, preventing phase separation. The effectiveness of these formulations when applied to the skin, however, relies on the ability of the gel matrix to deconstruct, enabling the separation of the oil phase from the aqueous phase. This has the impact of allowing the oils (emollients) to spread easily and form a uniform occlusive barrier over the skin surface, whilst prolonging emollient retention on the skin by rendering the oily ingredients resistant to re-emulsification (removal) upon subsequent contact with water (Ersser et al., 2007). The breakdown of the carbomer gel matrix is influenced by the shear applied, but driven by the interaction of the formulation with salts on the skin (Noveon, 2002). The interaction of the emollient gel formulations with salts is therefore an important consideration when occlusive performance of the product is of concern.

The purpose of this investigation was to assess the physical structure changes under the influence of salts and compare two marketed emollient gel products, namely Doublebase Gel (DBG) and Zerodouble Gel (ZDG) marketed in the UK.

Materials and methods

Visual appearance

Commercial samples of DBG and ZDG were dispensed into a container measuring 13 mm diameter by 1 mm deep and leveled using a glass slide. The samples were then allowed to stand at room temperature for 48 hours to allow evaporation of water.

Behaviour under the influence of salts

Nearly 10% w/w salt (NaCl) to gel was prepared by sprinkling 2.0 ± 0.1 g of NaCl onto 20 ± 0.4 g of each formulation and gently mixed by folding the sample on itself ten times using a spatula. They were then left to stand for

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30 minutes. Untreated control samples for each formulation were folded in the same manner without added salt solution.

Microscopy

Approximately 20 mg of each treated and control sample was mixed with Nile Red fluorescent dye. The samples were then placed on microscopic slides and pressed with cover slips for 5 seconds. After 1h, the samples were viewed under a Laser microscope (Nikon eclipse 90i) x 60 magnification.

Results and discussion

Considerable differences were observed between the surface characteristics and consistencies of the two formulations prior to salt treatment. DBG was found to have a smoother and a more homogeneous textural appearance when compared to ZDG. These differences were found to be even more evident on drying under ambient conditions. These differences in appearance could result from the manufacturing processes and the composition and quality of the ingredients used.

Carbomers are sensitive to the presence of salts. The addition of small amounts of salt can be used to thicken the emulsion. The thickening of the polymer is brought about by repulsion of like charges on the polymer backbone, causing the polymer to swell. Most carbomer grades have a tolerance limit of 0.1% salt (Noveon, 2002). In the presence of a higher concentration of salt, this repulsion is reduced, resulting in the collapse of the extended polymer chain and reduced viscosity. This behaviour of the polymer, in the presence of salt, is important for the delivery of the emollients during the application of these skincare products i.e. the salts on the skin are expected to break down the polymer, making the formulation spread more easily while releasing the emollient.

The behaviour of the two gel formulations after coming into contact with salt (NaCl) were found to be different. The DBG formulation seems to largely break down into a liquid (decreased viscosity). The ZDG formulation, on the other hand, does not break down and instead appears to curdle and become firmer (increased viscosity). This implies that the polymers used in the manufacturing of the products are not similar. Furthermore, this difference in behaviour demonstrates why the DBG formulation might be expected to spread more easily when applied to the skin.

Microscopic examination also revealed differences between the emulsions, both as original untreated formulations and following salt exposure. For DBG, the structural network stabilizing the oil droplets breaks down completely, releasing the oil droplets from the emulsion. In contrast, for ZDG, microscopic examination suggests that the emulsion structure does not break down to the same extent and manner as DBG.

Conclusion

Presented work has shown that simple visual inspection of the physical structures of two emollient gels before and after interaction with salts can reveal important differences between two products that may have implications for their effectiveness and patient acceptability. The two emollient gel formulations studied have quite different visual appearances and behave very differently in circumstances mimicking contact with salts on the skin. In contrast to ZDG, DBG is a homogeneous gel that completely breaks down in the presence of salts, releasing the oil droplets from the emulsion. This has very important implications in relation to clinical performance and patient preference, where the ease with which the oily ingredients can be spread, irreversibly delivering a uniform and occlusive barrier over the skin, are crucial. These results therefore point to qualitative differences between the formulations and very likely reflect important differences between their respective manufacturing methods and ingredients.

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Short communication

Emollient gels: Characterisation of textural properties and behaviour in the presence of salts

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Introduction

Topical leave-on emollient gel formulations are one of the most cosmetically acceptable therapeutic moisturisers, because of their non-greasy feel (Ersser et al., 2007). These oil-in-water (O/W) dispersions use carbomer as both the gelling and emulsifying agent. Whilst the carbomer acts to physically stabilise the dispersed oil droplets and thus prevent phase separation, its ability to deconstruct once applied to the skin is an important indicator of the effectiveness of these formulations. Deconstruction/breakdown of the gel matrix, due to contact with salts in the skin, results in phase separation of the oil from the aqueous phase, allowing the emollients (oils) to spread easily and form a uniform occlusive barrier over the skin surface. This also has the impact of prolonging the emollient retention on the skin by rendering the oily ingredients resistant to re-emulsification (Ersser et al., 2007).

When applied, the ability of the gel formulation to spread easily and leave a uniform occlusive layer of emollients on the skin is reliant on the textural properties i.e. firmness/stiffness of the gel matrix. These textural properties also have an important impact on the cosmetic acceptability of the formulation, such as the stickiness of the gel, which may influence the patient's willingness to generously apply the product, and therefore obtain the maximum benefits.

Interaction with salts causes the carbomer gel matrix to break down and it is therefore expected to have an important influence on the textural properties (Noveon, 2002). Characterizing the textural properties before and after interaction with salts could be an important indicator for the performance of the product during application.

The purpose of this investigation was to compare the textural properties of two marketed emollient gel products, namely Doublebase Gel (DBG) and Zerodouble Gel (ZDG) marketed in the UK, before and after interaction with salt (NaCl).

Materials and methods

Firmness/stiffness and Stickiness by Texture Analysis (TA)

50 g of commercial samples of DBG and ZDG were weighed into a beaker and subjected to compression using 35 mm diameter cylindrical probe (Stable Microsystems TA-HD plus) to measure firmness/stiffness and stickiness.

The force was measured as the probe compressed the sample by 15 mm distance after an initial trigger force (0.5 N) at a rate of 0.5 mm/sec.

When 15 mm target distance was reached the probe moved back to its starting position at 10 mm/sec recording the force required to separate the probe from the sample. This force is an indicator of stickiness. Samples were analysed in triplicates.

Spreadability by TA

1.1 ± 0.1 g of samples were compressed between two glass plates using predetermined forces, namely 1, 5, 20, 40 and 50 N. At each force the area of spread was marked out and calculated.

Different samples were used for the measurements of speadability at each force applied.

Behaviour under the influence of salts

Nearly 10% w/w salt (NaCl) to the gel were prepared by sprinkling 2.0 ± 0.1 g of NaCl onto 20 ± 0.4 g of each formulation and gently mixed by folding the sample on it-

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self ten times using a spatula. Firmness/stiffness, stickiness and spreadability of these samples were determined in the same manner as pre-treated samples.

Results and discussion

Firmness and stickiness of moisturisers are important sensory characteristics for consumers (Herman, 2007). Furthermore, firmness of the formulations contributes to the ability of the material to spread. These properties of the products were investigated using texture analysis, which allows for the determination of the force required to compress the formulation (a measure of firmness) and the force required to separate the probe from the sample (a measure of stickiness).

Considerable differences were observed between the two untreated formulations in terms of firmness and stickiness. ZDG appears to form a significantly firmer (3.6 ± 0.1 N) and more sticky (3.7 ± 0.2 N) polymeric structure in comparison to DBG formulation (2.6 and 2.5 N for firmness and stickiness respectively).

Upon treatment with salts, the DBG polymeric structure readily breaks down, resulting in extensive loss of firmness. In contrast, significant firmness of the ZDG structure was maintained after exposure to salts. The stickiness of the products could not be differentiated after salt treatment.

Important differences in spreadability were observed between the two emollient gels under applied force. The DBG formulation spread more easily than ZDG. For example at 20 N of applied force, the untreated DBG formulation spread over an area of 24.6 ± 0.1 mm2, whereas the ZDG formulation spread over an area of 16.2 ± 0.1 mm2. The capability of DBG to spread was greatly increased when exposed to salts and demonstrates a 27 % increase in area of spread. Interestingly, no such effect was observed for ZDG as no substantial difference was observed between the ZDG samples prior to and after being exposed to salts (15.4 mm2 area of spread obtained).

Conclusion

The presented work shows that these two emulsified gel formulations have different textural characteristics, with DBG being a less firm and less sticky gel that spreads more easily in comparison to ZDG. These two gels also behave very differently in circumstances mimicking contact with salts on the skin. In contrast to ZDG, the DBG polymeric structure readily breaks down when exposed to salts, resulting in extensive loss of firmness and increased ability to spread. The study data highlights important qualitative differences between emollient gel products, which may have resulted from differences in their respective manufacturing methods and ingredients. This may in turn, have important implications for clinical performance and patient preference. Furthermore, this type of study points out the need for more comprehensive analysis of products as patient and prescriber perceptions may lead them to select products that sometimes may not be as beneficial as they believe. Development of new methodologies for the assessment of emollient gel products is therefore necessary to fully assess the potential performance of such products.

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Short communication

Influence of diabetes and hypertension on cefuroxime permeation across placenta in pregnant women

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Introduction

The present study investigated the transcellular and placental permeation of cefuroxime, an antibiotic used in cesarean sections, in pregnant women with diabetes and hypertension. Previous studies have shown that infections after cesarean section could be decreased using preoperatively an antimicrobial agent (Chelmow et al., 2001). However in order to achieve therapeutic concentrations in both maternal and fetal serum and amniotic fluid, antibiotic have to pass placental barrier. It is proven that a single dose of intravenously administered antibiotic is as effective as multiple doses given perioperatively (Lamont et al., 2011). Accordingly, single doses of cefuroxime have been widely used for antimicrobial prophylaxis during cesarean delivery. Previous studies have shown that cefuroxime concentrations in amniotic fluid and in umbilical cord plasma are sufficient to combat most microorganisms. However, diseases such are hypertension and diabetes could alter placental transferto the extent that the prophylactic effect is lacking in both the mother and fetus. Therefore, the aim of this study was to determine the effects of diabetes and hypertension on the transplacental permeation of cefuroxime.

Materials and methods

Fifty-three women scheduled for cesarean section were divided into three groups: healthy women (n = 18), women with arterial hypertension (n = 21), and women with gestational diabetes (n = 14). All women received 1.5 g, intravenously cefuroxime. Study was conducted in accordance with international ethical guidelines (CIOMS) and the study protocol was approved by Ethics Committee of the Gynecology and Obstetric Clinic of Clinical Centre of Vojvodina (no. 100-08/9). Informed consent was obtained from each woman before enrolment in the study. The study was designed as an open-label study. Each intravenous injection of cefuroxime was completed in less than 1 min. Sampling points were chosen in order not to disturb regular cesarean section procedures in the clinic. Blood samples were collected from mothers after administration of cefuroxime before delivery (t_1), at the time of delivery (t_2), and after delivery (t_3). At delivery, umbilical venous and arterial samples were obtained from a section of umbilical cord (cross-clamped at delivery). Estimated gestational age at birth, weight, length and 1and 5 min Apgar scores were recorded. Concentration of cefuroxime was measured using a modification of an HPLC method described previously (Szlagowska et al., 2010). Pharmacokinetic parameters were calculated for each woman using plasma cefuroxime concentration data with WinNonLin version 4.1 (SCI software, Pharsight Corporation, Gary, NC, USA). The effects of diabetes and hypertension on cefuroxime placental-permeation were assessed by the fetomaternal plasma concentration ratios (Cv/Cm) and umbilical cord venous blood (Ca/Cv). Results were compared using ANOVA test ($p \square 0.05$).

Results and discussion

Neonates born to women in the hypertensive group had significantly lower body surface area (BSA), weight, 5-min Apgar score, and gestational age than neonates born to women in the control group. Neonate characteristics in the diabetic group did not differ significantly compared with either the hypertensive or control groups.

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Tthe Ca/Cv ratio in all three groups was near 1, with no significant differences among the control, hypertensive, and diabetic groups (1.01±0.16, 1.06±0.23, and 0.95±0.34, respectively). This correlates with the physicochemical characteristics of cefuroxime, which is a very hydrophilic weak acid (Log Poctanol/water 0.4) and is almost completely dissociated at blood pH (7.4) (Holt et al., 1993). Thus, the slightly acidic pH of neonatal compared with maternal blood would not lead to the accumulation of cefuroxime in neonates. In the present study, neither hypertension nor diabetes had any significant effect on cefuroxime accumulation in neonates. The Cv/Cm ratios were significantly lower in the diabetic compared with the control and hypertensive groups (0.36±0.13 vs 0.71±0.46 and 0.59±0.40, respectively). However, there were no significant differences between the control and hypertension group and because gestational age was significantly lower in the hypertensive than control group, the findings confirms those of a previous study that reported that gestational age had no effect on cefuroxime crossing the placental barrier (Holt et al., 1993). Pharmacokinetic parameters, such as drug plasma concentrations at zero time (C_0), mean resident time (MRT_0–t_3) and areas under the time-concentration curves to infinity (AUCinf) were not significantly different among the three groups. The elimination half-life ($t_{½}$) was significantly shorter in the hypertensive than control and diabetic groups because the constant of elimination (λz) was higher in the former group compared with the latter groups. Apparent volume of distribution and clearance were significantly lower in the diabetic group compared with the control and hypertensive groups. Lower transplacental transfer in the diabetic group compared with the hypertensive and control groups could be also due to lower volume of distribution and clearance.

Conclusion

Hypertension had no significant effect on the permeation of cefuroxime nor on its pharmacokinetics. Diabetes led to decreased placental transfer of cefuroxime, as well as volume of distribution and clearance, but did not affect other pharmacokinetic parameters. Prophylactic concentrations of cefuroxime were reached in all groups, but the dosing time of cefuroxime should not be less than 30 min or greater than 2 h prior to delivery.

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Placental transfer of lipophilic drug diazepam in pregnant women with diabetes and hypertension

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Introduction

Due to the widespread rise in early detection of high-risk pregnant women in need of cesareans, its use is rapidly increasing worldwide (e.g. uncontrolled diabetes, hypertension and pre-eclampsia). Diazepam is lipophilic drug that is weak base and it is used in the treatment of maternal eclampsia and as a premedication in cesarean section deliveries. Diazepam readily crosses the blood–brain barrier and the placenta by passive diffusion. It is also excreted into breast milk and studies showed that diazepam reaches equilibrium in the feto-maternal systemic circulation 10-15 minutes after intravenous administration (Bakke et al., 1992). Also, some diseases such as diabetes and hypertension have been associated to impaired placental composition and functions. Previous studies carried out in our laboratories have demonstrated impaired drug permeation in diabetic animals. The development and progression of diabetes have been associated with disturbed drug absorption due to dysfunctional protein expression and functionality, impaired transcellular transport and intercellular trafficking as well as altered gut physiology (Al-Salami et al., 2009). The aim of this study was to investigate the influence of diabetes and hypertension on the placental permeation of diazepam.

Materials and methods

Pregnant women were recruited from the Gynecology and Obstetric Clinic in Vojvodina, Serbia. Pregnant women scheduled for cesarean section, those who were diagnosed with gestational or arterial hypertension as well as those who were diagnosed gestational diabetes were included in this study. The study protocol was approved by Ethic Committee of the Gynecology and Obstetistric clinic in Vojvodina (N°00-08/9) and informed consents were obtained from each participant before inclusion in the study. A total 75 pregnant women were divided into three groups: group 1 (healthy control, n=31), group 2 (diabetic, n=14) and group 3 (hypertensive, n=30). Two sets of diazepam plasma samples were collected and measured (after the administration single dose of 5 mg/day intramuscularly), before (t_1), during (t_2) and after delivery (t_3). The first set of blood samples was taken from the mother (maternal venous). The second set of samples was taken from the fetus (fetal umbilical veins and arteries). Diazepam concentrations in plasma were measured by modified HPLC method previously described (Rouini et al., 2008). Pharmacokinetic parameters were calculated using non compartmental analyses using with WinNonLin version 4.1 (SCI software, Pharsight Corporation, Gary, NC, USA). Values of AUCs after delivery were taken as a measure of diazepam elimination from blood. In order to assess the effect of diabetes and hypertension on diazepam placental-permeation, the ratios of fetal to maternal blood concentrations were determined. Also umbilical cord arterial to umbilical cord venous concentration ratio was determined as a measure of diazepam uptake, distribution and/or metabolism in neonates. Data were analyzed by ANOVA test and differences were considered statistically significant if $p \leq 0.05$.

Results and discussion

All neonates were similar in length, weight and body surface area values. Also, there were no statistically significant differences neither in height, weight nor body surface

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area of the women between three investigated groups. The diabetic and hypertensive groups have 2-fold increase in the fetal umbilical-venous concentrations, compared to the maternal venous concentrations. Feto: maternal plasma-concentrations ratios were higher in diabetic (2.01±1.01) and hypertensive (2.26±1.23) groups compared with control (1.30±0.48) while, there was no difference in ratios between the diabetic and hypertensive groups. Umbilical-cord arterial: venous ratios (within each group) were similar among all groups (control: 0.97±0.32; hypertensive: 1.08±0.60 and diabetics1.02±0.77) and there were no statistically significant differences. There were statistically significant higher AUCs values before delivery in control and hypertension group compared to diabetes group. Meaning that transfer of diazepam in diabetic group was higher even though exposure was lower probably due to increased permeability of placenta in diabetic women. Values of AUCs after delivery were statistically higher in control group compared to hypertension and diabetes group, but there were no statistical differences between hypertension and diabetes group. These results implies that elimination of diazepam from central compartment is higher in hypertension and diabetes group and that is likely that there were more unbound diazepam in the blood in these groups, since total clearance of diazepam is directly proportional to free diazepam fraction (Riss et al., 2008).

Conclusion

On line with our previous findings which demonstrate disturbed transcellular trafficking of lipophilic drugs in diabetes, this study shows significant increase in diazepam placental-permeation in diabetic and hypertensive pregnant women suggesting poor transcellular control of drug permeation and flux, and bigger exposure of the fetus to drug-placental transport.

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Self-microemulsifying drug delivery systems containing simvastatin: formulation and characterization

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Introduction

Simvastatin is poorly water-soluble drug which belongs to class II (low solubility, high permeability) according to Biopharmaceutical Classification System (BCS) (Kuentz, 2012). One of the strategies to improve its dissolution rate includes development of self-microemulsifying drug delivery systems (SMEDDS). SMEDDS are isotropic mixtures of oil, surfactant, cosurfactant, and a drug, that under dilution in vivo can spontaneously form microemulsions with droplet size less than 50 nm (Gursoy and Benita, 2004; Hauss, 2007).

The aim of this study was to formulate and characterize self-microemulsifying drug delivery systems of simvastatin with increased dissolution rate of simvastatin.

Materials and methods

Materials

Simvastatin (Ph. Eur. grade) was obtained from Hemofarm a.d. (Serbia). Caprylocaproyl macrogol-8 glycerides (Labrasol®), propylene glycol monocaprylate (Capryol™ PGMC) and oleoyl macrogol-6 glycerides (Labrafil® M1944CS) were obtained from Gattefossé (France). Polysorbate 80 was obtained from Sigma Aldrich Chemie GmbH (Germany).

Methods

Formulation and preparation of SMEDDS

Surfactant phase of Labrasol® as surfactant and Polysorbate 80 as cosurfactant were mixed at fixed weight ratio 3:1. Oil (Capryol™ PGMC or Labrafil® M1944CS) was then added to surfactant phase at varios ratios (from 9:1 to 1:9) and mixed on magnetic stirrer. After preparation, all samples were titrated with highly purified water drop by drop. Two single-phase, transparent systems are considered to be microemulsions and selected for further investigations. Both samples contained 67.5% Labrasol, 22.5% Polisorbat 80 and 10% oil (Capryol™ PGMC-sample F1 or Labrafil® M1944CS-sample F2). Simvastatin (5% w/w) was dissolved in these selected SMEDDS by constant mixing on magnetic stirrer at 50-60 °C until a clear solution was obtained (samples F1s and F2s).

SMEDDS characterization

Droplet size determination

Both, unloaded and simvastatin-loaded SMEDDS were diluted with highly purified water (1:10). The average droplet size (Z-ave) and polydispersity index (PDI) of unloaded and simvastatin-loaded systems were determined immediately after dilution by photon correlation spectroscopy (NanoZS90, Malvern Instruments, UK) at wavelength of 633 nm and a scattering angle of 90 °. The results were the mean and standard deviation (S.D.) of three consecutive measurements for each sample.

In vitro dissolution studies

For *in vitro* dissolution studies liquid SMEDDS with 20 mg of simvastatin were filled into hard gelatin capsules (size 0), and compared to commercially available tablet containing the same dose of simvastatin.

The dissolution test was carried out using rotating paddle apparatus (Erweka DT70, Germany). The dissolution medium consisted of phosphate buffer pH 7.0, the volume

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was 900 ml, and the temperature of the dissolution medium was maintained at 37 °C with a rotating speed of 75 rpm. At fixed time intervals (5, 10, 15, 30, 45 and, 60 min), 10 ml samples were withdrawn from the dissolution medium and replaced by 10 ml of fresh phosphate buffer. Sink conditions were maintained at all times. All samples were filtered using membrane filter (0.45 μm MF-Millipore® membrane filter, Millipore Corporation, USA) and simvastatin concentration was determined spectrophotometrically at 239 nm (Evolution 300, Termo Fisher Scientific, England). The dissolution experiments were carried out in triplicate, and data were expressed as mean value ± S.D.

Results and discussion

Droplet size analysis

The average droplet size (nm) of samples F1, F1s, F2 and F2s were 18.58±0.04, 46.41±0.17, 11.68±0.12 and 17.45±0.04, respectively. It could be concluded that upon high water dilution both unloaded and drug-loaded SMEDDS are capable to form microemulsions, because the average droplet size is less than 50 nm (Gursoy and Benita, 2004; Hauss, 2007). Slightly higher droplet size of F1s, compared to unloaded F1, might be due to the interference of the drug with self-emulsification process (Gursoy and Benita, 2004).

Polydispersity index represents the uniformity of droplet size within the formulation and for selected samples F1, F1s, F2 and F2s were 0.155±0.005, 0.286±0.001, 0.137±0.016 and 0.168±0.005, respectively. Lower value of PDI in sample F2s indicated better uniformity of droplet size, in comparison to formulation F1s. Both unloaded and SIM-loaded SMEDDS have shown monomodal droplet size distribution.

In vitro dissolution study

Comparative in vitro dissolution profiles of simvastatin from SMEDDS filled in hard gelatin capsules and commercial (immediate release) tablet showed that simvastatin was completely released from both SMEDDS within first 5 minutes. The release rate of simvastatin from SMEDDS was significantly faster compared with commercially available tablet (approximately 14.57% after 1 hour), which might be due to the surfactants present in formulations. Karim et al. (2015) showed that the droplet size of the microemulsion could determine the rate and extent of simvastatin release, since the in vitro drug release was faster from formulations with smaller droplet size. However, in this study there was no significant difference in simvastatin release between two selected SMEDDS.

Conclusion

Self-microemulsifying drug delivery systems (SMEDDS) containing simvastatin were formulated and evaluated. Upon appropriate water dilution SMEDDS formed microemulsions with droplet size less than 50 nm. Although F2s had smaller droplet size and narrower droplet size distribution, in vitro dissolution study revealed that simvastatin was completely released from both SMEDDS (F1s and F2s) in 5 minutes. These results indicated that development of SMEDDS could effectively enhance in vitro dissolution rate of simvastatin compared to commercial tablet and can be used as possible alternative.

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A spectroscopic insight into the albumin structure on the nano-bio interface

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Introduction

In the last decades, the struggle for efficient targeted drug therapy and diagnostics paved the road to clinical use of nanomedicines. However, the research and understanding of the interactions among the engineered nanomaterials and the biological environment, to date, presents quite a challenge. It is becoming clear that, when placed into a biological environment, nanoparticles initiate a cascade of interactions with the biomacromolecules resulting in the formation of the 'protein corona' (a layer(s) of proteins adsorbed on the nanoparticles surface) (Monopoli et al., 2011). These interactions can alter the secondary structure of the adsorbed proteins promoting instability and/or exposure of new epitopes at the protein surface, thus giving rise to unexpected biological responses (Calzolai et al., 2010). Undoubtedly, the protein corona modifies the nanoparticles interface and thus affects their biological fate and overall performance. Therefore the characterization of the interactions at the nano-bio interface will greatly influence the understanding and capability for prediction of the nanoparticles in vivo behavior. The aim of this work is to investigate the effects of the surface properties of different polymeric nanoparticles upon their interaction with a model protein (bovine serum albumin - BSA) in a binary nanoparticle – BSA system.

Materials and methods

Materials

PLGA-PEO-PLGA (Mw 148KDa and Mw 22KDa) was purchased from Akina Inc (USA). Lutrol F127 - Poly(ethylenoxide)-block-poly(propyleneoxide)-block-poly(ethyleneoxide) was kindly donated by BASF (Germany) and BSA was purchased from Sigma Aldrich (USA). Bradford Protein assay dye reagent was obtained from Bio-Rad (USA). All other reagents and chemicals used were of analytical grade.

Methods

Nanoparticles preparation procedure

Nanoparticle formulations were prepared from PLGA-PEO-PLGA (Mw 70,000:8,000:70,000Da) – NP1 and PLGA-PEO-PLGA (Mw 6,000:10,000:6,000Da) – NP2, using the nanoprecipitation method, as described previously (Dimchevska et al., 2015).

Quantification of bovine serum albumin adsorption

All samples were diluted to concentration of 2mg/ml and subsequently 1ml from each formulation was mixed with 1ml of 2mg/mL BSA solution in phosphate buffer (pH 7.4). The NP dispersions with BSA were incubated for 1h at 37°C in a water bath with horizontal shaking at 100 min^{-1}. After the incubation, the samples were concentrated to 1mL using ultrafiltration tubes with pore size of 1000 kDa, and washed with phosphate buffer pH 7.4. Blank (BSA free) and control sample (without nanoparticles) were also used in the experiment. The amount of adsorbed BSA was indirectly quantified using the Bradford protein assay.

Spectroscopic characterization of nanoparticle-bovine serum albumin interactions

The samples were prepared as described above with additional freeze drying cycle (-40 °C, 0.055 mBar, FreeZ-

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one 2.5 L, Labconco, USA) in order to remove the water. FTIR spectra of the samples were carried out using FT-IR spectrometer (660, Varian, USA). To calculate the relative proportions of the different secondary structures of albumin, the spectral region of the Amide I (1700-1600 cm^{-1}) was analyzed with appropriate software for spectral analysis (Grams32, Thermo, USA). Using Gauss-Lorentz transformation, six distinctive peaks of the secondary protein structures were analyzed (β-sheets in spectral regions 1696-1690 cm^{-1}, 1642-1624 cm^{-1} and 1618-1613 cm^{-1}; β-turns in the spectral region 1685-1675 cm^{-1}; α-helix in the region 1658-1654 cm^{-1}; random coils in the region 1648-1640 cm^{-1}). To avoid any influence of the vibration of the polymer chains in the region of Amide I, the spectra of empty nanoparticles were previously subtracted from the spectra of the samples with adsorbed albumin.

Results and Discussion

The results from the quantitative BSA adsorption studies revealed that 24.6±1.9 and 13.1±0.9% of BSA were adsorbed on the surface of NP1 and NP2, respectively.

The FTIR spectra of freeze dried BSA revealed broad band at 3287 cm^{-1} that originates from N-H bending vibrations (Amide A). The C-H bending of aliphatic chains can be noted on 2960 cm^{-1}, 2933 cm^{-1} and 2875 cm^{-1}. Strong C-O stretching vibration (Amide I) that originates from the amide bond in the peptide chain appears at 1645 cm^{-1} while the Amide II band (mainly N-H bending vibrations) is on 1516 cm^{-1}. Several bands that represent mainly combinations of C-N stretching and N-H bending can be noticed in the region from 1400 cm^{-1} to 1200 cm^{-1}. Distinctive blue shift of Amide I (1654 cm^{-1}) in the spectra of NP1-BSA complex can be noticed, relative to the FTIR spectrum of BSA. Such shift of Amide I could be attributed to possible secondary structure changes of BSA that are initiated as a result of the interaction with the surface of the nanoparticles. Also, significant decrease in the C-O antisymmetrical stretching vibrations from the PLGA blocks was noticed, indicating to possible growth of hydrophobicity in the microenvironment of surface exposed carbonyl groups. The FTIR spectrum of NP2-BSA complex reveals blue shift of Amide I and II relative to the bands on BSA spectrum. Additionally, there is a slight decrease of the intensity of the carbonyl C-O vibrations band at 1756cm^{-1} and a red shift of the antisymmetrical C-O stretching vibrations on the PEO chains (1087 and 955cm^{-1}) relative to the respective bands in the spectrum of NP2. The curve fitting on Amide I in the previously mentioned FTIR spectra revealed that the secondary structure of the BSA molecule contains 35% α-helix, 32.5% β-sheets, 12% β-turns (short 4 amino acid segments that form antiparallel loops) and 20% of random coils. Having in mind that the spectrum was taken from freeze-dried sample of BSA, one can assume that the portion of α-helix will be reduced on the expense of the increase of β-sheets and random coils in the secondary structure. The NP1-BSA and NP2-BSA demonstrated presence of 77.63% and 34.39% of α-helix, 11.51% and 31.76% of β-sheets, 10.8% and 17.74% of β-turns, 0.05% and 16.11% of random coils in the BSA molecule, respectively.

Considering the above results one can assume that the behavior of BSA towards NP1 and NP2 is different, mainly because of the differences in the density and PEO chain length of the nanoparticle hydrophilic corona. The PLGA/PEO ratio in NP1 and NP2 is 17.5:1 and 1.2:1, respectively. Having this in mind, it can be presumed that the PEO chain length and surface density can affect the accessibility of the hydrophobic nanoparticle core towards nonspecific interactions with BSA. The hydrophilic corona of NP2 is larger and more rigid and as such will be an effective steric barrier in the interaction of BSA with the hydrophobic PLGA core. The loss of α-helix in the secondary structure of BSA in the native BSA sample and NP2-BSA complex can be attributed to the freeze-drying stress of the BSA molecule that initiates hydrogen bonding redistribution in the secondary structure of the protein. The partially adsorbed BSA molecule won't be able to protect its intramolecular hydrogen bonds during the freeze drying process that will initiate the conversion of α-helix to random coils and β-sheets. On the other hand, the structure of the BSA that is adsorbed on the NP1 surface could be associated with its' native structure. The preservation of the native structure during freeze drying is another indication of the hydrophobic nature of the NP1-BSA interactions resulting in significant contribution towards the maintenance of the BSA original hydrogen bonds.

Conclusion

The results unambiguously point to the effect of the hydrophilic outer nanoparticle layer as a steric barrier for nanoparticle-BSA interactions. The formulations with low hydrophilic coverage expose the hydrophobic core and enable strong hydrophobic binding with the proteins present in the corona. Such strong binding could result in appearance of different new protein epitopes on the nano-bio interface and significantly alter the biological fate of the nanoparticles.

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Short communication

Preliminary study concerning *Linum usitatissimum* oil as sebum-reducing agent

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Introduction

There are a lot of records in the literature regarding the utility of linseed oil on the skin. Concerning to this, it is worth to mention here the experimentally confirmed effects, such as increased skin hydration after introducing in the diet linum usitatissimum oil (De Spirt et al., 2009). The linoleic and alpha-linolenic essential fatty acids are involved in restoring the cutaneous barrier, important in skin ceramides synthesis. Other authors have noted the favorable effect of the linum usitatissimum oil on wound healing, on an animal model (De Souza Franco et al., 2012). There are also experimental evidences regarding the benefits on skin excoriations. Additionally, the anti-inflammatory and even antibacterial effect leads some authors to cite flax oil as showing a positive effect on treating ringworm.

On the other hand, theoretically, under the effect of lignans, (secoisolariciresinol diglucoside - SDG - the most abundant flax lignan) with estrogen-mimetic action, the oil of this species should have effects similar to other phytoestrogens. This is the premise of our experimental study.

The study aims to: (1) evaluate fatty acids in Linum usitatissimum oil, in order to be, at least, protective and nutritive for human skin, in topical application, (2) evidencing the sebum-reducing capacity of linum oil, (3) observation of a possible improvement of skin texture (a result of skin hydration), for the cases of seborrhea sicca (dry seborrhea).

Materials and methods

Linseed oil was obtained from the seeds of the Alexin cultivar grown in the Didactic Station Timisoara of Banat's University of Agricultural Sciences and Veterinary Medicine Timisoara in 2014. The cutaneous study benefited from the enrollment for testing of 24 healthy female volunteers, aged between 18 and 46, having oily and seborrheic skin and the sebaceous glands were measured in terms of numbers and dimensions, by using the apparatus Proderm Analyser (NU SKIN, Provo, UT, USA). The evaluation of the cutaneous evolution was performed in the Dermatopharmacy and Cosmetology laboratory (University of Medicine and Pharmacy Timisoara). The criteria for including the volunteers in the study were: (1) Written consent of the volunteers after they understood the test procedure, (2) Diagnostic of seborrhea or normal oily skin, based on clinical criteria, (3) Lack of any pathological complications (eg. acne, seborrheic scaling dermatitis) situations in which the oil is potentially harmful (obstructive, comedogenic, or irritating) (4) Elimination of intolerance, after a preliminary test of the oil at a topical skin application. The assessments with Proderm Analyzer were recorded after 7 days of therapy, at the times: 7, 14, 21 and 28 days respectively. For each volunteer, at each time point, were counted the colored points per skin field explored (number of oversized sebaceous glands per cm2) and the average was calculated for each time unit, separately for seboreea oleossa and for seboreea sicca.

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Results and discussion

Linseed oil is characterized by a high content of linoleic acid (0.313 mg / ml) and linolenic acid (0.214 mg / ml). The results are consistent with literature data mentioning a high concentration of linoleic acid in oil from linseed seeds cultivated in Romania but also in other countries. The daily use of linseed oil also produced an improvement in the appearance of the skin, reducing the appearance of scaly skin to complete fade-out. There are no obvious differences between the evolution of the seborrhea oleossa and the sicca during the test for the linum oil treated group, meaning that this oil has efficiency in both types of disorders.

The most significant decrease of sebaceous glands number was registrered starting with the 14th day after the first treatment. The 14 days also represent the turnover of the sebocyte. In other words, after two weeks there are other new sebocytes holding secretory activity under another hormonal stimulation, modified in the meantime. As a matter of fact, the sebocyte is a cell with a prompt response to other pharmaceutical treatments as well, for example after the treatment with topical retinoids the sebaceous secretion decreases dramatically, sometimes after only 7-10 days. The problem with retinoids, however, is their adversity to the epidermis, dryness and the erythema generated.

Conclusion

In conclusion, linseed oil is one of the oldest natural remedies for skin nutrition. Recent studies show its benefits in scarring, in surgical wound healing optimization, in skin hydration and also in the domain of cutaneous aging prevention.

This study brings again linum usitatissimum oil in the foreground, this time for its sebum-reducing effect. This property is a confirmation of the hypothesis launched by the Lucas Meyer dermocosmetic laboratories, a property attributed to the lignans in flax seeds, known for their estrogen-mimetic and implicitly anti-androgenic attributes, which means exactly an intervention on the hormonal mechanism of seborrhea. Our study opens a new prospect to lignans determinations, in different sort of linseeds, for a natural alternative in sebum-regulation field.

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Safety profile assessment of cosmetic anti-age creams based on natural ingredients using in vivo bioengineering techniques

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Introduction

In the last years, there is a growing trend of utilization of natural cosmetics, that is related to the frequent occurrence of allergies and skin irritations caused by synthetic ingredients commonly included in the formulation of these products (Glampedaki and Dutschk, 2014). Antioxidants, especially the ones naturally derived from plants, have been recognized as excellent source of bioactive components with beneficial effects on skin, especially to prevent premature skin aging and wrinkling. There is an increasing research interest in polyphenolic compounds which have proven to possess antioxidant activity, and have shown promising effects applied as cosmetic active ingredients (Braunlich et al., 2013).

Materials and methods

Therefore, our group developed an anti-age cosmetic line consisting of day (D) and night (N) cream, both based on natural materials. Namely, in the stated creams (D and N) black chokeberry and hibiscus propylene glycol extracts (produced at Institute for Medicinal Plant Research) and hyaluronic acid (low weight, Amedeo Brasca, Italy), were incorporated as active ingredients in the appropriate hydrophilic (D-V, yielding cream D) and hydrophobic (N-V, yielding cream N) vehicles, in which synthetic excipients were replaced with the ones of natural origin. Chokeberry is one of the richest sources of natural antioxidants among fruits and vegetables, because of its bioactive components such as anthocyanins, flavonols, procyanidins, and phenolic acids (Kulling and Rawel, 2008; Rugina et al., 2012; Sueiro et al., 2006). Beside chokebery extract, D and N creams contained hibiscus extract, abundant in vitamin C that stimulates synthesis of collagen, a protein responsible for skin tone and elasticity (Aburjai and Natsheh, 2003). Hyaluronic acid, natural component of the skin located in the extracellular matrix, recovers lost skin moisture and gives the visual effect of skin lifting (Scott and Banga, 2015). Avocado oil, grape seed oil and shea butter (all from Comcen, Serbia) included in both creams, effectively feed and revitalize the skin due to the complex of active ingredients they contain, especially high content of fatty acids. Creams contain components of natural wetting factors of skin (complex of hexylene glycol, fructose, glucose, sucrose, urea, dextrin, alanine, glutamic acid, aspartic acid, hexyl nicotinate) and elastin (both from Chemisches Laboratorium Dr Kurt Richter GmbH, Germany). Elastin as a protein, is responsible for skin tone, gives it back skin moisturize, elasticity and freshness. Both creams contain a complex of UV filters (ethyl hexyl methoxycinnamate, butyl methoxydibenzoylmethane, ethyl hexyl salycilate, Coning PPI, Serbia) that protect the skin from harmful sun rays.

Safety profile/irritation potential of the developed cosmetic products (active creams-D and N, as well as their matching vehicles-D-V and N-V, respectively), was evaluated using noninvasive measurements of the appropriate biophysical parameters of the skin in a 24-h in vivo study under occlusion.

Thirty one healthy female volunteers (mean age 46.09), which participated in the study, were thoroughly informed about the possible treatment effects and the protocol of the examination prior to signing written consents, in accordance with the Helsinki Declaration. The study was approved by the Ethical Committee of the Institute for Medicinal Plant Research "Dr. Josif Pančić", Belgrade, Serbia (Decision No 01-9337-13). The following param-

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eters were evaluated: electrical capacitance (EC) (quantifying the stratum corneum hydration), transepidermal water loss (TEWL), as a measure of skin barrier function and erythema index (EI), as a measure of skin colour, using Multi Probe Adapter MPA®9 (Courage & Khazaka Electronic GmbH, Germany). All measurements were conducted on flexor aspects of forearms at square application sites of 9 cm^2, leaving a site per each arm for untreated control under occlusion (UCO) and without occlusion (UC). The volunteers were instructed not to use dermopharmaceutical and/or cosmetic products on the tested areas as well as to spend at least 30 minutes in a room in which the measurements were conducted in order to adapt to the temperature and relative humidity. After initial measurements, 0.016 g/cm^2 of the investigated samples were applied, covered with silicone film and fixed with hypoallergenic adhesive tapes. Two hours upon removal of the 24-h occlusion, all parameters were reassessed (Jakšić et al., 2012; Tasić-Kostov et al., 2011).

Results and discussion

The investigated samples showed overall satisfying preliminary safety profiles (low in vivo irritation potential). Namely, two hours after occlusion removal, all the investigated samples led to the significant upsurge of EC compared to the baselines and controls, revealing skin hydration potential probably related to appropriate vehicles themselves (D-V and N-V), bearing in mind lack of significant differences after treatment with these samples compared to the matching active creams (D and N, respectively). There was no significant change in EI, which was even decreased for all the tested creams, indicating well-tolerated skin formulations. Also, there was no significant increase of TEWL for the investigated samples compared to the baseline values, nor UCO. Significant growth of this parameter was detected after repeated measurement for the sample N compared to UC, but this increase cannot be attributed to the treatment with this sample itself, while the same difference between test-spots N and UC was also noted in the initial measurement.

Conclusion

In conclusion, preliminary safety profile of the investigated creams based on ingredients of natural origin can be considered satisfactory. Tested samples did not cause the change in the measured biophysical parameters-TEWL and EI, while they even increased EC i.e. stratum corneum hydration, a feature considered preferable in the cosmetic products intended for the treatment of aged skin.

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Short communication

Small-scale production and evaluation of an acetate-and a lactate -based balanced infusion solution

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Introduction

Intravenous (IV) fluid therapy is a commonly used in hospitalcare practice as one of the essential element in patient care. Over the years IV fluids in different forms have been specially designed and developed according to the physiological demands of various patient's medical conditions. Understanding these IV fluids that are administered to the patient is important because each has a differentimpact on the body and particular indications for use. Therefore, the choice of IV fluid should be based upon the hydration status of the patient and metabolic disorders associated with the patient's condition. .

Ringer's solution for infusion is chemically prepared an isotonic crystalloid that contains sodium chloride, potassium chloride, and calcium chloridein sterile water. The dosage of Ringer's solution is dependent upon the age, weight, clinical conditions of the patient, and concomitant therapy.

This solution is indicated to replace extracellular fluid losses, to restore the sodium, potassium, calcium and chloride balances and, as well as for treatment of isotonic dehydration condition In practice, there are different recipes for Ringer's solution composition depending on its intended use. For example, lactated Ringer's solution is a hypotonic solution that the best approximate extra cellular fluid. It may be infused safely in large quantities in patients with conditions such as hypovolemia with metabolic acidosis, shock syndromes and burns. It is well known that lactate is metabolized in the liver, and to a lesser degree inthe kidney while, acetate is metabolized mainly in the muscles and lesser in tissues such as kidneys and heart, so it's a good alternative in patients with impaired lactate clearance such as in advanced liver disease (Santoro et al., 2007; Zander, 2004). In general, there are three independent acid-base variables that need to be determined when studyingthe acid-base properties of IV fluids such as: the partial CO_2 tension, the total concentration of nonvolatile weak acid (AToT), and the strong ion difference (SID) (Kellum, 2002, 2005). Metabolic acidosis and alkalosis are respectively caused by raising and lowering AToT while holding SID constant. Metabolic acidosis and alkalosis are respectively caused by lowering and raising plasma SID while clamping AToT. Fluid infusion causes acid-base effects by forcing extracellular SID and A ToT toward the SID and A ToT of the administered fluid (Morgan, 2005). The SID of isotonic saline being 0, the infusion of large quantities (as in correction of hypovolaemia, acute normovolaemic haemodilution, and cardiopulmonary bypass) will dilute the normal SID of plasma and decrease pH. Using the Stewart equation, a balanced solution with a physiological SID of 40 mEq/Lwould induce a metabolic alkalosis (Chappel, 2008; Morgan, 2005). In order to avoid this inducti; n, balanced solutions using organic anions such as lactate, acetate etc. increases the SID and also decreases the osmolarity of the solution (Bertrand, 2010; Chappel, 2008). Morgan has calculated that a balanced solution should have a SID of 24 mEq/L (Bertrand, 2010; Morgan, 2005).

In the field of surgery and intensive care, hyperchloremic acidosis is well-known problem in patients receiving large amount of standard electrolytes. A series of studieshas emphasized the disadvantageous effects of hyperchloremic acidosis on various organ systems, for example, hemodynamics, NO-production, renal blood circulation, urinary output or hemostasis.

Having in mind that there is a lack of Ringer's lactate and Ringer's acetate solution on the drug market in our country, the aim of presented work was to formulate these IV solutions, and to evaluate theirquality and stability. Prepared solutions, were used in our Hospital in the Department of Anesthesiology.

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Material and methods

The minimum conditions required for the small scale production and quality assurance of Ringer's lactate and acetate solutions were established by elaboration of the required Pharmacopoeias monographs (Ph.Eur.8th; USP 39). The IV fluids were prepared in laminar flow cabinet (LFC), using an aseptic technique along with sterile filtration and filled in the sterile containers. Solutionswere sterilized by autoclaving. The final solutionswerethen submitted to quality control,

Results and discussion

Prepared IV fluids had good quality in respect to physical properties, physico-chemical parameters and microbiological quality according to Ph.Eur. 8. It was also confirmed that IV fluids were stable for a year in the conditions of the second (II) climatic zone. Each of these IV solutions had a different profile in terms of impact on acid-base status, electrolyte levels, coagulation, inflammation, renal, and liver function. The choice of the best solution for patient resides in a complete understanding of the expected response of each solution and the patient 's risk factors.

In the preliminary comparison study, Ringer's lactate as well as acetate- based IV solution, proved to be suitable for fluid replacement during surgery. Hemodynamic stability remained unaffected by both of the solutions. Concerning consistency of acid base parameters none of the solutions seemed to be inferior.

Conclusions

It has been revealed that prepared formulation of Ringer's lactate and Ringer's acetate solutions can be successfully used for fluid replacement.

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Distribution coefficient of gliclazide as *in vitro* prediction model of blood brain barrier penetration

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Introduction

Development of effective *in vitro* model for prediction of blood brain barrier (BBB) penetration of drugs is nowadays widely researched. BBB is selective barrier that eclipsed the brain and isolates it from the circulating blood. It represents a major barrier for drug permeation, especially of those molecules that are highly hydrophilic, ionisable, contain more than 10 hydrophilic moieties, low logP value and molecular mass bigger than 400 Da. Distribution of drug in system octanol/water is in correlation with its lipophilicity and its optimal value ranges between 2.5 and 5 (Pardridge, 1998). Most of newly developed drugs have poor permeability. Thus novel pharmaceutical formulations are developed containing drug permeators. Drug transfer across BBB could be facilitated using permeator enhancers (e.g. bile acids). Value logP in system octanol/water is dependent on drug solvent interaction (i.e. hydrogen bonding), and because of this it is a poor predictor for BBB penetration, since this barrier is highly hydrophobic. Better system is cyclohexane/water since cyclohexane has no possibility for hydrogen bonding. If permeator that is most suitable could be predicted *in vitro* in preformulation investigations, it could decrease formulation development costs. Thus the aim of this study was to investigate gliclazide distribution in systems n-octanol/water and cyclohexane/water as *in vitro* prediction models for *in vivo* BBB penetration. Also the aim was to determine whether mentioned *in vitro* models could predict the influence of permeators such is dexycholic acid (DCA) on gliclazide transfer across BBB.

Materials and methods

Distribution coefficient (logD) was determined using a "flask shake" method. In glass tubes 1 ml of organic solvent (cyclohexane or octanol) was mixed with 5 ml of aqueous phase. Partition profile was determined over physiological pH range (pH 1.2 HCl solution, pH 4.5 acetate buffer, pH 6.8 and 7.4 phosphate buffer and pH 7 distilled water) for 5 combinations of n-octanol or cyclohexane with aqueous gliclazide solution (10 µg/ml) of different pH and with or without the addition of DCA (0.5 mM) into n-octanol or cyclohexane phase. The analyses were done in triplicate for each pair of organic solvent/water. Concentrations of gliclazide were determined using modified high performance liquid chromatography method (Mikov, 2008). Values of logD at pH 7.4 were compared with literature date of gliclazide BBB penetration and influence of DCA on its penetration (Lalic Popovic, 2012).

Results and discussion

Gliclazide is a small lipophilic molecule which is expected to readily cross biological barriers. However it has poor BBB penetration, and since it shows antioxidant properties on brain cells its penetration into central nervous system is of interest (Sandoval, 2009). Though some drugs do cross BBB, there is a great number of drugs like gliclazide for which BBB is impermeable. In this investigation a higher partition into organic layer was found in system n-octanol/water than cyclohexane/water. Profiles of distribution after 1 and 24 h were different which leads to a conclusion that 24 h is needed time for a partition to be finished in system cyclohexane/water. There were no observed differences in logD values after 1 or after 24 h between systems with and without DCA in n-octanol (pH

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6.8; 7 and 7.4), but minor differences were noticed in lower pH values (i.e. pH 1.2 and pH 4.5). Also DCA significantly increased partition of gliclazide in system cyclohexane/water but not in system n-octanol/water. Value of logD at pH 7.4 without DCA in organic layer in system n-octanol/water was 0.34±0.05 and in system cyclohexane/water was -0.74±0.11. According to logD value in cyclohexane/water, gliclazide have poor BBB, which is in correlation with *in vivo* data (logBBB 0.23±0.02 healthy animals and 0.85±0.03 diabetic animals) (Lalic Popovic, 2012). Value of logD at pH 7.4 with DCA in organic layer in system n-octanol/water was 0.54±0.07 and in system cyclohexane/water was 0.18±0.01. Both systems showed increased transfer of gliclazide when DCA was present in organic layer. This is also in correlation with *in vivo* investigation where animals were pretreated with DCA (logBBB 0.96±0.03 healthy animals and 1.35±0.14 diabetic animals) (Lalic Popovic, 2012). Thus partition of gliclazide in system cyclohexane/water better correlates with *in vivo* data where penetration of gliclazide was increased with DCA pretreatement, but in diabetic animals, penetration was increased in group with and without DCA pretreatement and investigated *in vitro* systems could not this predict.

Conclusion

Increased logD value in organic layer when DCA is present indicates existence of physicochemical interactions of DCA and gliclazide. Investigated system cyclohexane/water predicted poor gliclazide BBB penetration and the influence of DCA on gliclazide penetration but system n-octanol/water failed to do so. However investigated systems could not predict differences in penetration between diabetic and healthy animals. Further investigations are necessary to determine value of system cyclohexane/water in prediction of BBB transfer.

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Choosing the right blister packaging film

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Introduction

The main function of packaging is to ensure protection of the pharmaceutical product, its safety and proper use, information on the identity, instruction of use and storage at any time point before expiration date of the drug product. The pharmaceutical dosage product and its container closure system act together to serve as an integral unit. The primary packaging comes into direct contact with the product and it is the most significant component in the pharmaceutical product packaging. It serves the two main functions of protecting the product from outside influences that would otherwise render it useless while allowing the manufacturer of the product to package it using practices that typically involve automated form-fill-seal equipment. As innovations continue and new films enter the market, a thorough understanding of how regulatory, package performance and film attributes fit together should help ease the selection process and, hopefully, provide some framework to avoid potential problems throughout the life of the pharmaceutical product.

CGMP requirements for packaging materials for parenteral, inhalation and liquid products are wider than for solid oral dosage forms (FDA, 1999).

Objective

Correct selection of contact packaging has a significant impact on safety and efficiency. The goal is to choose the appropriate contact material for blister packaging. Principles and contribution to simplification and proper choice of packaging material are: out of the vast number of packaging materials to choose a range of materials that can be used for packaging a certain product, materials that meet quality requirements and are tested by the manufacturer, from reliable suppliers of high quality and favorable price, to fully comply with the equipment and resources for packaging at the factory. An analysis of the characteristics of materials for blister packaging was made.

Critical factors in choosing the right blister packaging film

For choosing the right blister packaging film it is necessary to have great knowledge of drug characteristics, physical and chemical properties, microbiological quality, technical considerations of blister films, dimensional stability of the webs, ability for thermoforming and cooling of formed film, variation in thickness, compatibility of forming film and lidding foil.

Films for blister packaging

The base in blisters produced by thermoforming process is a polymer plastic film. The blisters are formed under increased temperature. PVC film used for blister packaging represents a rigid film due to the absence of softeners and plasticizers, which provides structural rigidity and physical protection of the pharmaceutical dosage form. It is transparent, rigid material with a greattermoform ability, easy to color and low cost. The main disadvantage is low barrier properties, high water vapor and gases permeability. PVDC has a significant role in blister packaging as lamination or coating of PVC. PVDC provides excellent barrier to gases and water vapor, unlike other polymers that provide either one or other protection. Permeability to gases does not depend on the relative humidity, so it can be used for packaging in various environmental conditions. The properties of the copolymer depend on the content of the VDC, greater amount of VDC result in better barrier properties, the smaller the amount of VDC the flexibility improves. The amount and type of co-monomer, as

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well as additives and manufacturing technology affect the properties of PVDC. The weight of PVDC coatings ranges from 40, 60, 90 and 120 g/m^2. PVDC coatings have been used with duplex (PVDC/PVC) and triplex (PVDC/PE/PVC) structure being the most common ones used (Bauer, 2009). Aclar® has the lowest water vapor permeability of all the films used for blister packaging. It has good barrier properties to gases, but this material is not used often in applications where impermeability to gases is primary demand. Aclar®UltRx (CTFE homopolymer) has the highest moisture barrier of any clear thermoplastic film. It processes within the same range as other thermoforming films on conventional blister packaging equipment. PET has a higher permeability to water vapor compared to PVC. PVDC - coated PET has a similar permeability to water vapor as PVDC. PS has very good thermoforming properties, but has high permeability to water vapor. PP has good barrier properties to water vapor (similar to PVDC). The problem is thermoforming. The temperature required for thermoforming PP and the further cooling process must be precisely controlled. There may be distortion of blisters in secondary packaging due to the thickness of PP film. Another disadvantage is the thermal instability and the possibility of collection of the film after performing the process. The process of blister packaging on a standard blister machine with a PP is difficult to perform and is much slower unlike PVC. COC (Cyclic Olefin Copolymer) in multilayer combinations with PP, PE or PETG has very good barrier properties. Cyclic olefins have good thermoforming properties even for deep blister cavities. COC is used in combination with semicrystal PE or PP polymers to improve thermoform properties. The films are produced with co-extrusion or lamination. COC does not contain halogen in molecular structure comprising only from carbon and hydrogen. COC is used for packaging which requires deep blister cavities (Pilchik, 2000).

Blisters produced by cold forming are formed mechanically without the use of temperature and have high barrier properties. OPA/aluminum/PVC/ALU has excellent barrier properties for oxygen and is also impermeable to water vapor and therefore, it is the first choice of packaging material. Its cost per square meter can withstand comparison with PVDC. However, cold forming takes up more packaging material for the same number of tablets or capsules of the same size, unlike PVDC.

Multilayer films can be laminated, co-extruded, or a combination of the two. In laminated films, all film layers are extruded separately and are bonded together by a thermoset adhesive. In co-extruded films, the film is manufactured in a single-step operation in which the film layers are bonded together by a tacky thermoplastic polymer. These two manufacturing techniques can be combined. Flexible materials are used for the layers of the laminates, such as aluminum foils and films of polymer materials of varying thickness. With the right choice of a layer, a laminate with best suited properties for the packaging of a product at a low price could be obtained. Foil for the inner layer usually has good barrier properties, the lowest permeability to gases and water vapor. The inner layer should not have interaction with the product. The possibility of shaping and closing is provided by inner layer that should be termosealable (PE, PVC, PS, PVDC) (Stagnaro, 2011).

Conclusion

The categorization of the polymer film is made according to their barrier properties: WVTR (water vapor transmission rate) and permeability to gases. PVC has a significantly higher permeability to water vapor from all types of PVC/PVDC and all types Aclar®. PVC/PVDC and Aclar® are closely comparable values for WVTR, depending on the thickness of the impermeable layer in the polymer film. Gas permeability, especially O2 and CO2 is important when the product inside the packaging material is sensitive to oxidation. PVC and all types of Aclar® have significantly higher oxygen permeability of all types PVDC film. OPA/aluminum/PVC/ALU has excellent barrier properties for oxygen and is also impermeable to water vapor.

Identifying the most suitable blister packaging material is complex. It requires very good understanding of the film properties in relation to application/ machine parameters for getting an optimal packaging performance.

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Short communication

Qualification of cleanrooms in pharmaceutical industry

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Introduction

Clean rooms and associated controlled environments provide control of airborne particulate contamination to levels appropriate for accomplishing contamination-sensitive activities. Production of sterile pharmaceutical products is regulated in a separate Annex 1 in European Guideline for Good Manufacturing Practice (EU GGMP, 2008) and contains instructions for minimizing the risk of contamination of these specific products with particles, microbes and pyrogens.

Presented study represent a critical review of some tests in guidance document for pharmaceutical industry, used in the process of qualification and monitoring of clean rooms for the manufacture of sterile medicinal products.

Qualification of clean rooms

Qualification of clean rooms in pharmaceutical industry is comprised of different tests like air supply, air velocity, air changes, flow pattern, filter integrity, pressure test, particle count, temperature, microbial count, relative humidity, noise level and vibration test.

The purpose of air supply capacity test is to demonstrate that the air system is balanced and capable of delivering sufficient air volumes as per design to maintained required air change in the defined area. The air capacity is demonstrated by following the procedure of measuring air flow in supply and returned duct and air volume to meet the design required.

The purpose of air velocity/uniformity test is to present the capability of the air system for delivering sufficient air volumes to maintain a minimum cross section velocity under HEPA terminal filter modules. The measuring is performed by calibrated anemometer at numerous sites in order to provide one measurement for each 0.37 m^2 filter area. For Laminar Flow sections air flows uniformity has to be 0.45 m/sec ± 20%.

Testing of air flow pattern is performed in order to check the interference due to turbulence eddies in unidirectional airflow area, like sampling and dispensing booth and under laminar airflow in microbiological area (Kitain, 2010) Visualizing the air patterns at numerous points in room is performed by Titanium Tetra chloride sticks. In several cases the test is done by operating the HVAC system of the sterile area and releasing smoke into unidirectional air stream at selected sites. There is no minimum GMP requirement for air changes per hour. Air flow into and out of a space should be based on providing the required cooling, heating, relative humidity, pressurization, particulate control, ventilation. These factors generally result in air change rates between 4 and 20. There is also no numerical requirement for relative pressurization in cleanroom. The velocity and direction of airflow between spaces should be adequate to reduce counter flow of airborne particulates or vapour contaminants for spaces where airborne cross contamination is a concern (EU GGMP, 2008).

All HEPA filters installed in the facility are tested for filter integrity test by using PAO (Poly-alpha-olefin) aerosol into supply duct to the HEPA filter. Sampling of stream challenge is done with photometer and the instrument is set for this challenge. Cross contamination can originate from both the internal and external facility environment. In all air handling systems, the filtration should be evaluated for adequate arrestance of outdoor particulates. Any suitable particle counter instrument can be used for the measurements, with no effect of the measurement principle used. During the operation an air flow rate should be 0.03 m^3/min. Measurement should be done at minimum 10 different representative room locations for one minute at each location at 1 meter height from the floor. It must cover the central location of personal traffic during normal production process (WHO, 2011). Measurements should obtain

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information about concentration of two different ranges of particles: particles with size ≥ 0.5 μm as well as particles with size ≥ 5 μm (5 μm particles for ISO 5 class has been excluded from the limit value table), at each location (DIN EN ISO 14644-1, 2015; DIN EN ISO 14644 - 2, 2015).

Room temperature variation often can be a critical parameter in production processes. Most products, materials and processes can handle a wide range in temperature. The width of this range decreases as the exposure time increases. Existing HVAC system had designed for 24 ± 2 °C to all critical area in manufacturing, warehouse and Quality Control department. Sometimes, for some products, in-processing temperature requirement shall be maintained 18 °C ± 2 °C. Relative humidity may affect exposed product or materials that are sensitive to air moisture. Relative humidity generally has effect on aqueous product. Liquid product can lose moisture to a low humidity room over an extended period. HVAC system in sterile area is designed to maintain the required humidity. Relative humidity is checked by using calibrated humidity meter from different locations (DIN EN ISO 14644-1, 2015; DIN EN ISO 14644 2, 2015).

Microbial monitoring of manufacturing clean rooms should include compressed gases, surfaces, room and enclosure air and any other materials and equipment that may produce a risk of contamination. Monitoring of the air should be performed on meter cubic air (active or passive sampling) by using special instruments or settle plates which are opened for 4 hours and after that incubated. Surface sampling is conducted by using swab or contact plat, collecting the microbiological contamination from approximately 25 cm^2 surface from numerous defined locations. Taking samples for microbiological monitoring of the area should be performed for a period of one year routine production in accordance with the sampling plan. Noise level may be present in the facility due to operation of variety of equipment, during processes etc. Requirements when personnel noise exposure exceed an eight hours time weighed average sound level of 85 dBa. HVAC system has designed not to generate more than 70 dBa noises in critical area during its normal operation (Kitain, 2010).

Conclusion

Clean environments should be certified as described in ISO 14644 series in order to meet their design classification requirements. The design, construction, and operation involved in clean rooms and advanced aseptic clean rooms operations vary greatly, so it is difficult to generalize requirements for parameters such as filter integrity, air velocity, air patterns, air changes, and pressure differential. In particularly critical applications such as aseptic processing, a structured approach to physical risk assessment might be appropriate.

Situations where some of the parameters are out of range with values defined by guidelines of pharmaceutical products manufacturing undoubtedly are reason for deviation. After researching the presumable causes, the goal is finding solution for solving the problem, its future prevention and reseting the qualified conditions of the cleanroom.

Clean-room operators, particularly those engaged in aseptic processing, must strive to maintain suitable environmental quality and must work toward continuous quality improvement of personnel operations and environmental control. In general, fewer personnel involved in aseptic processing and monitoring, along with reduction in interventions, reduces risk from contamination.

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Effect of formulation and process variables on probiotic viability after microencapsulation by spray-drying in soy protein-alginate microparticles

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Introduction

Consumption of probiotic products has been linked to improvement of a variety of health conditions such as hypercholesterolemia, hypertension, inflammation and lactose intolerance and reducing health risks, including allergy and even some forms of cancer. However, for probiotics health benefit to be exerted, it is estimated that they should be administered in a therapeutic minimum of 10^{-6} to 10^{-7} CFU live probiotic cells per g final product (FAO/WHO, 2006). Providing and maintaining at least this number of viable cells is a great challenge due to their sensitivity to the harsh processing and GI conditions prior taking their place of action in the low intestine and delivering the claimed health benefits. For incorporation of probiotics into food or pharmaceutical products, the microencapsulation offers protection to the fine particles produced during freeze- or spray-drying of probiotic concentrates. Among the many techniques for microencapsulation, spray-drying is one of the most challenging because of the low cost, industrial application, stability and throughput in cellular integrity while drying when optimized correctly. However, the spray-drying process parameters might have negative impact on the outcome of viable cells. In addition, the choice of carrier material and its interaction with the bacteria have importance on protection efficacy and affect the probiotic delivery. In search for novel probiotic microencapsulated formulation that will combine favorable properties of alginate (ALG) and polymer that will provide controlled and targeted release of viable probiotic cells in the lower intestine, we have encapsulated the probiotic *L. casei 01* in ALG and soy protein isolate (SPI) matrix by spray-drying. The aim of this study was to evaluate the effect of the critical formulation and process variables on viability of the probiotic after spray-drying.

Materials and methods

FD-DVS/*Lactobacillus casei 01* was supplied from Chr. Hansen (Copenhagen, Denmark), SPI from Sojaprotein AD (Becej (Serbia), whereas ALG (10/60 LS, fG 35%–45%) was kindly donated from Protanal FMC Biopolymers (Ayrshire, UK). $CaCl_2$, de Man, Rogosa, Sharpe agar and broth as well as peptone water were purchased from MerckKGaA (Darmstadt, Germany).

Aqueous mixture of ALG and SPI was inoculated with bacterial suspension (cell load ca. 12.5 $\log_{10}$ CFU/g), activated as previously described (Petreska Ivanovska et al., 2014; Smilkov et al., 2014). The resulting mixture was infused into a spray-dryer nozzle unit of Büchi Mini Spray Dryer B-290 (Büchi Laboratorius-Technik AG, Switzerland) and continuously sprayed at following conditions:

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nozzle diameter 0.7 mm, aspirator pressure 90%, atomizer pressure 600 Nl h^{-1}, flow rate 5 mL/min. SPI-ALG microparticles (MPs) with encapsulated *L. casei* that were further subjected to additional cross-linking by $CaCl_2$ were prepared by consequent introduction of the MPs obtained by spray-drying into aqueous solution of $CaCl_2$, followed by continuous stirring at room temperature (1 h). MPs thus obtained were removed from the solution of $CaCl_2$ by centrifugation (1000 rpm, 5 min), washed three times with sterile water, frozen at -20 °C and freeze-dried at 0.070 mbar and -50 °C for 24 h (Freeze-Dryer, Labconco, USA). Critical material attributes (concentration of ALG, SPI and $CaCl_2$) and process parameters (inlet temperature, IT) were previously identified varying one parameter at time, while the influence of critical variables on viability of the probiotic after preparation was evaluated using face centered CC-RSM design (Design-Expert® V8, Stat-Ease, Inc., USA). The total of 30 experiments were designed and carried out, with the following actual levels of studied variables: ALG (A, 1 and 4%w/v), SPI (B, 1 and 4%w/v), $CaCl_2$ (C, 0 and 5%w/v) and IT (D, 90 °C and 150 °C). Viability of the encapsulated *L. casei 01* in SPI-ALG MPs was assessed after dissolution of 1 g MPs in 9 g PBS (1 mol/L, pH 8.0), using the plate-count method as previously described (Petreska Ivanovska et al., 2014; Smilkov et al., 2014).

Results and discussion

Viability of *L. casei 01* in designed formulations after preparation was in range of 8.67-13.09 $\log_{10}$ CFU/g or expressed in % related to initial *L. casei 01* cell count from 68.38 to 99.61%. Influence of examined variables upon this response in terms of coded factors was described by reduced cubic model with the following equation:

Viability(after preparation) (%) = + 86.78 + 0.95 x A - 2.36 x B + 1.78 x C - 3.29 x D + 1.76 x A x C + 3.72 x A^2 - 6.84 x A^2 x C

From the equation one can clearly see that the viability after preparation positively correlated with the factors A, C, AC and A^2 and inversely with B, D, and A^2C. With increase in ALG and $CaCl_2$ concentration, the viability of the probiotic increased, while oppositely was observed with increase in concentration of SPI, suggesting competition between the probiotic and SPI for the same bonding sites in ALG molecules. According to the literature data (Rajam et al., 2012), modification of the compactly folded protein molecules from their native form allows SPI-ALG interactions *via* hydrophobic and electrostatic interactions and hydrogen bonding as well. At the same time, a trend to segregation of SPI and ALG into separated microdomains can occur, which has also been confirmed when whey protein as complexation agent was used (Smilkov et al., 2014). One way ANOVA indicated that variable D is significant model term, meaning that increase in the IT significantly decreases probiotic viability after preparation. The loss of probiotics during thermal processing is the main disadvantage of MPs production by spray drying and it is related to cellular injuries (e.g. denaturation of DNA and RNA, damage of ribosomes, dehydration of cytoplasmic membrane, lipid peroxidation and rupture of cell membrane) due to water removal resulting from the combined effect of heat and mechanical stress (Soukoulis et al., 2013). Thus, protection from the IT of the spray-dryer, as a critical point in this method of microencapsulation, is required. In addition, the probiotic bacteria must be alive at the time of consumption of the product and also capable of reaching the large intestine in quantities that are sufficient to facilitate colonization and proliferation. Therefore, probiotic loaded MPs should be customized in respect to their physicochemical and biological/biopharmaceutical properties as well to make them suitable for incorporation into food or pharmaceutical product as well as for administration in healthy individuals and/or individuals with specific disease.

Conclusion

L. casei 01 loaded SPI-ALG MPs were prepared using spray-drying method, with viability of the probiotic after preparation significantly exceeding the minimal therapeutic value. Process and formulation parameters should be further optimized to obtain probiotic MPs with both, high viability after preparation and in simulated GI conditions.

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Short communication

Preparation of curcumin loaded nanoparticles: physicochemical characterization and in vitro evaluation

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Introduction

Curcumin is the active principle of the spice turmeric, produced by the rhizome of Curcuma longa (Zingiberaceae), which is widely used in traditional eastern medicine as a hepatoprotective, anti-infectious and anti-inflammatory remedy (Shehzad et al., 2010). A compelling body of recent evidence has shown that curcumin is endowed by pleiotropic antineoplastic effects, due to modulation of NFkB and other cell signaling pathways, implicated in cell survival, apoptosis and angiogenesis (Shehzad et al., 2010). Regretfully, the enormous therapeutic potential of curcumin can`t be exploited in clinical practice, due to its extremely unfavorable physicochemical and pharmacokinetic characteristics, and also due to the instability in systemic circulation (Singh and Khar, 2006). The contribution is focused on newly-synthetized octopus-shaped macromolecules, consisting of hydrophobic calix[4]arene core and four arms of hydrophilic poly(ethylene oxide) chains as platform for delivery of curcumin.

Materials and methods

Two methods for preparation of inclusion complexes were used:

Heating method described by (Loftsson et al., 2005) with slight modifications. Briefly, to aqueous solutions of increasing concentrations of CX[4]PEG polyoxyethylatedtertbuthylcalix[4]arene) (2 mg/ml – 12 mg/ml) a constant amount of curcumin (1 mg/ml) that exceeded its aqueous solubility (11 ng/ml) was added. The vials were closed and heated at 50 °C for two hours. After that, the samples were left at room temperature for 24 h. Then the samples were subjected to centrifugation at 5000 rpm for 10 minutes. The clear transparent supernatants containing the inclusion complexes were collected and the amount of the curcumin was analyzed using a validated UV/VIS spectrophotometric method at 427 nm.

Solvent evaporation method: series of samples containing a fixed concentration of curcumin (1 mg/ml) and increasing concentrations of CX[4]PEG (2-12 mg/ml) were prepared in absolute ethanol, and evaporated to dryness using a Buchi rotation-type vacuum evaporator (R-215, Sigma-Aldrich). The concentrations of CX[4]PEG were chosen on the basis of its critical micelar concentration (CMC) of 7.7 mg/ml (or 0.24 μmol/ml) (Momekova et al., 2012). Thereafter the dried CX[4]PEG/curcumin containing films were hydrated with deionizaed water and were left for 2 h at 50 °C and then in dark at room temperature for 24 h. Then the samples were centrifuged for 10 minutes at 5000 rpm. The transparent yellow supernatants containing the curcumin-CX[4]PEG complexes were analyzed for curcumin content using a validated UV/VIS spectrophotometric method. Phase-solubility profiles were obtained by plotting the solubility of drug versus the excipient concentration.

Characterization of the CX[4]PEG-curcumin complexation

UV/VIS spectroscopy

The UV/VIS spectra of curcumin (in absolute ethanol and 10% ethanol solution) and its CX[4]PEG complex (in deionized water) were recorded on JASCO V570 UV-Vis-NIR spectrophotometer equipped with thermostatic cell holder (Huber MPC-K6 thermostat with precision 1 °C).

Fourier transform infrared (FT-IR) spectroscopy analysis

Samples of pure curcumin, pure CX[4]PEG, their physical mixture, and a lyophilized complex were characterized by an IRAffinity-1 Shimadzu FT-IR spectrophotometer. The scanning range was between 4000 and 400 cm-1.

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Dynamic light scattering (DLS) analysis

The size, size distribution patterns and zeta-potential of curcumin loaded supramolecular CX[4]PEG aggregates were investigated by ZetaSizer NanoZS (Malvern Instruments), equipped with a 633 nm laser. The parameters were evaluated from the measurements at the scattering angle of 175 ° at 25 °C.

In vitro *curcumin release*

The *in vitro* curcumin release from supramolecular BEC-X aggregates was evaluated by regular membrane dialysis at 37 °C against phosphate buffered saline (PBS). 1 ml of tested formulations was placed in dialysis membrane tubing (MWCO 10,000). The dialysis bag was then placed in a temperature controlled vessel, containing 100 ml of PBS (pH 7). At various time intervals aliquots were taken from the released medium and assayed for curcumin by UV–VIS spectroscopy.

Results and Discussion

Phase solubility studies

The phase solubility studies of curcumin with BEC-X were performed using the procedure utilized for the evaluation of cyclodextrin inclusion complexes by Higuchi and Connors (Higuchi and Connors, 1965). Due to their amphiphilic nature, polyoxyethylated calyx(4)arenes (CX[4] PEG) can self-associate in water by forming well-defined spherical nanoparticles. At concentration below the CMC, CX[4]PEG drastically increased curcumin solubility by formation of inclusion complexes with high stability constant (Kc). A significantly higher solubility enhancement of curcumin was observed at concentration exceeding the critical micellar concentration, attributed with additional solubilization of curcumin into the hydrophobic domains of the supramolecular aggregates by non-covalent interactions.

UV/VIS characterization

In order to characterize the spectral behavior of curcumin and its inclusion complex, absorption spectra of pure curcumin in absolute ethanol and 10% ethanol are compared with the absorption spectrum of the inclusion complex in water. The characteristic absorption peak of curcumin at 427 nm is identical in the three media under investigation, which demonstrates that the inclusion complex is formed by non-covalent hydrophobic interactions. An interesting finding is the appearance of a shoulder at 361 nm in the spectrum of pure curcumin dissolved in 10% ethanol which cannot be seen in spectra of curcumin in absolute ethanol and in the inclusion complex of curcumin in water. The shoulder can be attributed to the shifting of the tautomeric equilibrium from keto–enol to diketo-form.

FT-IR analysis

FT-IR spectroscopy is a useful tool for characterization of inclusion complexes. Characteristic combination of a sharp peak at 3508 cm^{-1} and a broad peak at 3293 cm^{-1} in the curcumin spectrum implies the presence of aromatic OH group stretching vibrations (Kolev et al., 2005) and the intensive sharp peaks at 1626 cm^{-1} and 1601 cm^{-1} corresponding to mixed C=O and C=C vibrations and symmetric aromatic ring (C=C) stretching vibrations, respectively, did not interfere with the vibrations in BEC-X spectra and can be used as marks for description of curcumin in inclusion complex.

DLS analysis

Physicochemical characteristics of the nanoparticles (size, size distribution and zeta potential were evaluated by DLS and the results revealed particles of app.180 nm with monomodal distribution (PDI below 0.2) and zeta potential of – 20 mV suitable for systemic application.

In vitro *curcumin release*

The in vitro curcumin release profiles from supramolecular CX[4]PEG aggregates were studied under simulated physiological conditions for different incubation periods from 2, 4, 6, 8, 10 and 24 hours. The results showed initial burst release of curcumin, followed by slower drug release.

Conclusion

Thus on the grounds of the excellent in vitro biocompatibility profile and the favorable physicochemical and drug loading characteristics of the tested liposomal nanoparticles, and their ability to retain the intrinsic pharmacological properties of encapsulated drug they could be considered promising drug delivery platforms for lipophilic curcumin.

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Assessing the risk of alcohol-induced dose dumping: diclofenac sodium case

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Introduction

Coadministration of alcohol beverages with extended-release (ER) oral dosage forms may lead to more rapid drug release and altered systemic exposure. Therefore, recent trends in the development of ER dosage forms highlight the importance of the investigation of potential alcohol-induced dose dumping (ADD) (Anand et al., 2011; Lennernäs, 2009). FDA and EMA guidances include general recommendations regarding the assessment of ADD, stating that suitable *in vitro* dissolution tests should be carried out to identify the risk of ADD, and differentiate between rugged and vulnerable formulations (EMA, 2014; FDA, 2016). In addition, the International Pharmaceutical Excipients Council Europe (IPEC) working group on ADD has summarized relevant scientific and regulatory information to help pharmaceutical companies to better handle ADD issues. It has been recognized that increased solubility of drugs or excipients in the presence of ethanol, and/or formulation factors (e.g. impaired ability to retard drug release), in conjunction with changes in physiological conditions after alcohol intake (e.g. delayed gastric emptying) are the key factors affecting the kinetics of drug release from ER oral formulations. Knowledge about these factors can help to identify and develop ADD-resistant formulations.

The purpose of this study was: (i) to evaluate different dissolution test setups to *in vitro* assess the effect of ethanol on dose dumping from ER tablets, and (ii) to evaluate the potential of the combined *in vitro-in silico* approach for the prediction of drug absorption profiles after concomitant alcohol intake, using commercially available diclofenac sodium ER tablets as model formulations.

Materials and methods

Diclofenac sodium solubility was tested in various media (0.1 M HCl pH 1.1 and USP buffers pH 6.8 and pH 7.4 without/with addition of 40% ethanol). Drug dissolution from the investigated hydroxypropyl methylcellulose (HPMC)-based tablets (ER1 - Diklofen® 100 mg diclofenac sodium ER tablets, Galenika a.d., ER2 - Diklofenak 100 mg diclofenac sodium ER tablets, Hemofarm a.d.) was tested under different experimental conditions: (i) in paddle apparatus at 50 rpm, using single medium pH 7.5 (USP Test 1 for diclofenac sodium ER tablets) without/with addition of 5% or 40% ethanol, and (ii) in basket apparatus at 100 rpm, using media change method (pH 1.1 without/with addition of 5% or 40% ethanol for 2 h, pH 6.8 for 2 h, pH 7.4 for 20 h). The later setup was designed to simulate changes in physiological conditions as the drug travels along the gastrointestinal tract, and to approximate conditions in the stomach, since ethanol is mostly absorbed through the gastric mucosa. The obtained dissolution data, incorporated in drug-specific absorption model (Simcyp® Population-Based Simulator, v. 14.1; Certara™, USA), were used for *in silico* simulations of drug plasma concentration-time profiles. Drug physicochemical and pharmacokinetic properties, used as inputs for absorption modelling, were obtained from available literature sources or *in silico* estimated.

Results and discussion

The solubility study results indicated that solubility-limited drug dissolution from the investigated tablets can be expected only in medium pH 1.1 without ethanol. In other words, due to increased drug solubility in the presence of 40% ethanol, concomitant intake of strong alcohol beverages might induce dose dumping from diclofenac so-

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dium ER tablets even at low pH in the stomach.

Dissolution data revealed that 5% ethanol in medium pH 7.5 had no significant effect on drug release rate from the investigated tablets (similar dissolution profiles). In addition, drug release profiles from the two products were similar in medium pH 7.5 with 0% and 5% ethanol. The addition of 40% ethanol in medium pH 7.5 affected drug release rate from ER2 formulation, resulting in approximately 4 times shorter mean dissolution time in comparison to the drug release profile in simple buffer. On the other hand, diclofenac sodium release under "media change" conditions was not affected by the exposure to either 5% or 40% ethanol in acidic medium (less than 10% of drug dissolved in 2 h; similar dissolution profiles). Considering these results, the observed difference in alcohol-resistance of the investigated products in medium 7.5 with 40% ethanol is not expected to have significant effect on drug release profiles in vivo.

The simulated pharmacokinetic parameters, based on the selected input data set, including drug dissolution rate under "media change" conditions without ethanol (Cmax 0.50/0.55 µg/ml and AUC 4.12/3.91 µg/ml h for ER1 and ER2 tablets, respectively), were in agreement with the reported data from clinical studies (Altman et al, 2015). These results indicate that the employed *in vitro* dissolution test conditions for diclofenac sodium ER tablets could be considered biorelevant. As expected based on dissolution data, the presence of ethanol in acidic medium had no effect on drug absorption profiles (the simulated pharmacokinetic parameters were Cmax 0.41-0.58 µg/ml, and AUC 3.97-4.16 µg/ml h). Simulation based on the hypothetical dissolution scenario, illustrating the "worst case", demonstrated that 100% diclofenac sodium release from 100 mg ER tablets in the stomach would alter the rate and extent of drug absorption (Cmax 2.56 µg/ml, tmax 2.16 h, AUC 6.43 µg/ml h), and consequently, the formulation would lose its modified release characteristics. But even this scenario would not pose safety issues for the patients, since the simulated plasma concentration profile is in the therapeutic range (Altman et al, 2015).

Conclusion

The presented case demonstrate that *in vitro* dissolution testing using the proposed "media change" experimental setup could be indicative of drug *in vivo* behaviour in the presence of ethanol. In addition, the combined *in vitro-in silico* approach may provide insight into the effect of ADD on drug clinical performance, and therefore, can serve as an alternative to clinical studies for ADD risk assessment. This approach should be encouraged, and applied to other ER oral drug products.

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Small scale production of gel with menthol, benzocaine and procaine HCl

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Introduction

Antirheumatic and analgesics drugs are the most commonly used drugs to treat diseases of the musculoskeletal system and to alleviate all sorts of pain that are caused from different origins. Their systemic use causes long series of side effects, so combination therapies of drugs with systemic and local action or only medicinal products intended for topical use wherever possible, especially in physical therapy, would be treatment of choice.

Various formulations of dosage forms, by composition and consistency, allow different therapeutic approach, suitable for achieving the desired therapeutic effect and for being capable to adapt to the physiotherapist treatment.

According to the regulations of the pharmacopoeia or to other regulations, the galenic drugs are made in small batches in a galenic laboratory, which are intended to be administrated directly to a pharmacy or a health institution.

According to the health requirements, the preparation of pharmaceutical dosage forms in a galenic laboratory, mainly aims to provide a dosage form, which is designed as an opportunity for changes in the composition and consistency of the product. Therefore, it can be provided a product with a modified properties, compared with a finished and fixed composition of the medicinal and an auxiliary substances, or with the already existing traditional products, which have the same composition of active components, satisfying the needs of the healthcare professionals who work with them and also allowing a simultaneous comfort and a therapeutic effect for the patients.

The usage of this drug, immediately after the preparation, reduces the need for adding more funds to stabilize and to ensure longer shelf life.

Obtaining the quality of this drug product, that satisfies the standards and maintains or enhances the therapeutic properties, requires appropriate conditions for a preparation and knowledge of all the components properties, technological process and requirements for the finished product as well.

The usage of the traditional liquid, a pharmaceutical product known as "Russian water", which contains: alcohol, menthol, procaine hydrochloride and benzocaine, is well received and accepted by the patients and the physiotherapists, but it also has some negative sides, such as, short effect, it is easily volatile, it acts surface and cannot be recommended as a massage supplement. On the other hand, gels are a dosage forms for external use that can be applied easily, to tie up to the skin and to enable the drug to heal the affected area,it can realize deep action, such as the ability to quickly penetrate multi-layers on the skin, to be easily rinsed from the site of application and generally does not irritate the skin, which is largely satisfying for our requirements.

The aim of this study was to formulate and prepare gel formulation from the pharmaceutical composition named "Russian water", with the ingredients: menthol, benzocaine, procaine HCl, which is easy and pleasant to apply on the painful area, also effective to reduce the pain and the inflammation. Formulation challenge was slight solubility of some of the components, getting a stable product with a proper consistency, ensuring the release of the active ingredients, their affection on the skin and a prolonged action.

Materials and methods

In the formulation and preparation the following active substances were used: menthol (Alkaloid), benzocaine

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(Sigma Aldrich), procaine HCl (Sigma Aldrich), at the recommended doses and ingredients, carbopol 940 (Sigma Aldrich), glycerol, (Alkaloid), triethylene tetramine (Sigma Aldrich) and aqua distillate.

Gel with menthol, benzocaine and procaine HClwas prepared in the galenic laboratory in Ohrid -General Hospital.Used ingredients and a compounding procedure described in a rational manner are:

menthol 4.0, benzocaine 2.0, procaine HCl 2.0, base gel ad 200.0. Basic gel was made with the following proscription:Carbopol 940 2.0, glicerolum 7.5, triethylene tetramine 2.5, aqua destilata 190.0.

In the step 1, preparation of base gel, Carbopol 940 and aqua distillate 50 mL were left to bulge. Then glycerolumwas gradually added and mixed to achieve good homogenization. After that, triethylene tetraamin was added and mixed again until thick gel was obtained.In the step 2, preparation of healing gel, the menthol was grinded through a sieve, and it was measured in the prescribed amount. It was mixed with the same amount of prepared basic gel to complete uniformity of the mixture. The benzocaine was grinded, sieved and measured at the prescribed amount and mixed with equal quantity of earlier prepared gel with menthol.

The prescribed amount of procaine hydrochloride was measured and dissolved in distilled water 10mL. Then, the solution was gradually added to the gel with menthol and benzocaine, whileconstantly stirred.The remaining amount of distilled water was added, gradually and with constant stirring, to the homogeneous gel to obtain a homogeneous product.

Results and discussion

The obtained gel is a homogeneous product with milk appearance. The high degree of pulverization of the components should improve solubility and ensure uniform distribution of insoluble components in the mixture. The process of mixing equal amounts of the basic mixture and pulverized substance also provides uniform distribution of the components, and the high density of the base gel reduces the possibility of their sedimentation (Allen, 2002; Jovanovic and Sekulovic, 1987; Racev and Lambov, 2005; Simov et al., 2001; Winfeld and Richards, 1998). Menthol is practically insoluble in water and because of its sole distribution in the preparationit was pulverized and a special way for its incorporation into the system was applied.Benzocaine is poorly soluble in water and an appropriate pulverization and mixing improved solubility and its uniform distribution in the product.The procedure of mixing and gradual addition of a small amount concentrated procaine hydrochloride solution reduces the possibility of decomposition of the gel structure under the influence of the potential ionic solution (Jackson and Lowey, 2010; Simov et al., 2001).

The obtained gel is packaged in plastic recipients of 200 mL, closed with a suitable stopper and signed with red signature marked “For external use.”It is used topically by applying to the affected area and/or with good rubbing in physical therapy.The product has a short shelf life and is prepared immediately before use, and to ensure the quality of the product it is recommended to be kept at a room temperature in well-closed recipients.During application it gives pleasant cooling sensation and pain relief and easily rinse out with water. It is not toxic and does not cause skin irritation.

The product can be prepared in galenic laboratory, and easy application and efficiency makes it acceptable to patients (Jackson and Lowey, 2010). It has been retained at the administration site longer than the expected, and has longer actions regarding to the liquid alcohol solution, which is with the same components.

Conclusion

The results of the treatment of patients according the investigation of the staff that operated and the practical patients show that this gel is with a prolonged analgesic activity compared to the liquid preparation, with an easy and safe application, a pleasant cooling sensation and a pain relief, also an easy flushing and with an increased satisfaction of therapists and patients.

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Short communication

Approaches in evaluation of freeze-dried antibody conjugates

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Introduction

Antibodies, proteins and other biotechnological products are often challenging in terms of their in-solution stability. Various physical and chemical changes occur during their in-solution storage, leading to shorter shelf-life. Freeze drying is often proposed as a method of choice, since removal of water has been reported to greatly reduce the rate of degradation, both in physical and chemical terms. Still, during freeze-drying antibodies and other protein pharmaceuticals can experience in-process changes that may reduce their physicochemical biological and/or pharmacological properties. In this context, many attempts have been made to reduce these changes, both in optimization of the freeze-drying process and in optimization of solution formulation, adding various buffers, cryoprotectants, etc.

The presented experience was in freeze-drying of monoclonal antibody – rituximab, conjugated with three types of bifunctional chelating agents, p-SCN-Bn-DOTA, p-SCN-Bn-DTPA, and 1B4M-DTPA, and evaluation of possible changes in post-freeze-drying phase. In order to assess possible defragmentation of the antibody, protein integrity test was performed, using SDS-PAGE electrophoresis and the analysis of several structural elements of FT-IR and Raman spectra pre- and post- freeze-drying process, provided an insight in possible changes in the structure.

Materials and methods

Commercially available rituximab (Mabthera®) was conjugated with three bifunctional chelating agents, p-SCN-Bn-DOTA, p-SCN-Bn-DTPA, and 1B4M-DTPA (Macrocyclics Inc. USA). The conjugates were synthesized, purified, adjusted to concentration of 1 mg/mL and freeze dried, using Labconco Free Zone Stoppering Tray Dryer (USA), as previously described (Smilkov et al., 2014). The protein integrity was assessed using SDS-PAGE that was performed in about 5 μL of reconstituted samples and 1 mg/mL purified, commercial rituximab (Mabthera®). Samples were mixed with sample buffer and boiled 5 min at 95 °C. Approximately 5 μL of each preparation was applied in 12% bisacrylamide under reducing conditions. Visualization of the bands was enabled using Coomassie Brilliant Blue R-250 (Sigma, USA). For comparison, low molecular weight marker (Amersham GE Healthcare, UK) was used.

For determining protein structure, FT-IR spectroscopy was conducted on PARAGON 1000 (Perkin Elmer, USA) spectrophotometer in the spectral range 2000–500 cm^{-1}. Attenuated Total Reflectance (ATR) spectra were acquired at a resolution of 4 cm–1. The obtained data was processed with Grams_32 software (Thermo Scientific). Raman spectra (2000–400 cm^{-1}) were recorded on a micro-Raman multichannel spectrometer Horiba JobinYvon LabRam 300 Infinity, using He:Ne laser. The spectral resolution was set to 4 cm^{-1}. The acquisition time and the accumulation number were set to 10 s and 10 scans, respectively (Gjorgieva Ackova et al., 2015).

Results and discussion

Using SDS-PAGE electrophoresis it is possible to determine the purity and, therefore possible defragmentation. The electrophoresis in reducing conditions resulted in two distinct Mw species which migrated in two bands in all

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three rituximab conjugates. The upper band corresponded to ~50 kDa and the lower band to ~25 kDa. This migration pattern is characteristic of IgG antibodies that have two identical subunits, each composed of two polypeptide chains: two heavy and two light chains, linked via four disulfide bonds. Under the action of the reducing agent DTT, the antibody is separated to heavy and light chains, with molecular weight corresponding to the two formed bands (Maleki et al., 2013; Nebija et al., 2011). The lyophilization protocol used did not affect structure properties and caused no post-lyophilization modification, as shown in the reducing SDS-PAGE lane patterns, compared to commercially available rituximab sample.

Spectroscopy studies can reveal information to witness preserved secondary structure upon freeze-drying, a mandatory prerequisite for immunoconjugates. Protein denaturation upon lyophilization is usually monitored by IR spectroscopy (Murphy et al., 2012), although Raman spectroscopy can also be applied (Wen, 2007).

The IR spectra of all three rituximab conjugates revealed higher percentage of β-sheet conformation (antiparallel and parallel) in the structure (strong band in the region between 1612 and 1640 cm^{-1}, followed by a weaker band around 1685 cm^{-1}), followed by α-helices (bands at 1655 or 1656 cm^{-1}), as obtained in the band frequencies for amide I, II and III bands which are used as diagnostic bands. We observed that the freeze-dried rituximab conjugates regain their native conformation upon rehydration (reversible unfolding).

Thermally-induced aggregation processes of the majority of proteins can also be studied by FT-IR and Raman spectroscopy. Strong absorption bands below 1620 cm^{-1} can be correlated with aggregation, usually associated with the formation of new strong beta-sheet structures (Schüle et al., 2007). With the lowest frequency band detected at 1620 cm^{-1} (in all samples analyzed), we concluded no obvious aggregation in all three freeze-dried antibody conjugates.

Conclusion

Among the many techniques available for evaluation of the structure and stability of freeze-dried antibody conjugates, SDS-PAGE electrophoresis, FT-IR and Raman spectroscopy can be employed in assessing structural properties as well as in determination of stability of these potential drug candidates.

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Short communication

An injection method for preparation of liposomes as ketoconazole carriers

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Introduction

The drug delivery with a specified rate in the body for a certain period of time has become an important tool for improving therapeutic index (Patel et al., 2009). Reducing toxicity to the health cells and potential drug degradation is important for achieving an effective treatment (Chanda et al., 2011; Kaur and Kakkar, 2010). Liposomes are considered to be excellent models of cell membranes, as well as for stabilizing the pharmaceutical active substances (Chanda et al., 2011; Kaur and Kakkar, 2010). Phospholipids and cholesterol assembled in one or more lipid bilayers with an aqueous core are the main components in the microscopic spherical liposome vesicles (Chanda et al., 2011). The specific characteristics of the liposomes such as non-toxicity, flexibility, targetability to specific cells or tissues, and biodegradation make them drug carriers, reducing drug toxicity through encapsulation (Kaur and Kakkar, 2010; Patel et al., 2009). The importance of their utilization is due to the ability to encapsulate hydrophobic, hydrophilic, and amphiphilic active pharmaceutical substances (Gómez-Henz and Fernández-Romero, 2006; Sahasrabuddhe et al., 2012). Generally, liposome formulations are classified based on their structural characteristics, substances in their composition, size and lamellarity, method of preparation, and their application (Kaur and Kakkar, 2010; Sahasrabuddhe et al., 2012).

Ketoconazole (cis-1-acetyl-4-[4-[[2-(2,4-dichlorophenyl)-2-(1H-imidazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl]piperazine) is a chiral lipophilic imidazole antimycotic drug administered mainly as a racemic mixture (50:50) of enantiomers in the cis configuration. The decomposition of the drug could be easily caused through acidic, chemical, photolytic, and oxidative conditions. Commercially available ketoconazole pharmaceutical dosage forms such as topical cream, antidandruff shampoo, ointments, and tablets possess anti-inflammatory and some antibacterial activities (Patel et al., 2009; Sahasrabuddhe et al., 2012). Potential nephrotoxicity, hepatotoxicity, and decomposition of ketoconazole require preparation of new safety formulations (Patel et al., 2009). In the ketoconazole liposome preparation, a thin-film hydration (Patel et al., 2009) and an injection method using either chloroform (Sahasrabuddhe et al., 2012) or dichloromethane (Patel et al., 2009) were described. The potential adverse effects of alkyl halide solvents (WHO, 2004) emphasize the aim of this work for introducing less toxic solvent such as methanol in ketoconazole liposome preparation through injection method.

Materials and methods

Standard ketoconazole was received as a gift from Pharmaceutical Company Replek Farm DOOEL–Skopje, Republic of Macedonia. Analytical grade methanol, HPLC grade methanol and water were supplied from Merck (Germany). Cholesterol was supplied from Calbiochem (Japan) and L-α-phosphatidylcholine (egg yolk) was from Sigma (Germany). Potassium hydrogen phosphate and potassium dihydrogen phosphate were supplied from Alkaloid AD (Republic of Macedonia). An analytical balance Mettler Toledo (Switzerland) was used for the sample

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weighing with 0.1 mg accuracy. A magnetic stirrer (type MM–530 Tehnika Železniki, Slovenia), a rotary evaporator (Devarot Elektromedicina, Slovenia), and a centrifuge (MRC, Pharmachem, Republic of Macedonia) were used in the ketoconazole vesicle preparation. An Agilent Technologies 1200 high-pressure liquid chromatographic (HPLC) system (USA) equipped with a diode array detector (G1315D), a binary pump (G1312A), a degasser (G1379B), a thermostatted column (TCC G1316A), and an autosampler (ALS G1329A) were used for the encapsulation efficiency determination of ketoconazole in liposome formulations. The chromatographic separation was achieved on a column LiChrospher®100 C-18 (150 mm length x 4.6 mm i.d., 5 μm particle size) with a mixture of methanol and water (90:10 v/v) mobile phase adjusted to pH 8.90 with a phosphate buffer. The absorbance was recorded at 296 nm. The morphology of ketoconazole liposomes was determined with an optical microscope Konus, (type M–100–FL, Italy), equipped with a digital camera (Sony, Cyber–shot W, type DSCW830V.CE3) and a lens ZEISS Vario–Tessar 8x (China).

Ketoconazole, L-α-phosphatidylcholine, and cholesterol in the weight ratio of 3.3:1:3.3 w/w/w were transferred with 5 mL methanol in a 100 mL round-bottom flask. Following the homogenization process on the magnetic stirrer at 1000 min^{-1}, methanol was evaporated in the rotary evaporator at 55 °C and 80 min^{-1} (10 min). The lipid mixture was added to a portion of 5 mL distilled water (80 °C). The ketoconazole liposome mixture was left overnight in order to mature at 4 °C. The separation of the liposome and free amount of ketoconazole was done in the centrifuge at 5000 min^{-1} for 10 min.

Results and discussion

In the preparation of ketoconazole liposomes through injection method, the solubility of ketoconazole was considered as a criterion of choosing less toxic methanol instead of chloroform (Sahasrabuddhe et al., 2012). The large unilamellar ketoconazole liposome vesicles were prepared using the injection method with methanol. The determined encapsulation efficiency of the ketoconazole liposome formulations in methanol of 45% was smaller in comparison to the encapsulation efficiency of the ketoconazole in chloroform (71%) obtained by Sahasrabuddhe et al., 2012. The smaller encapsulation efficiency was justified with harmless preparation conditions of ketoconazole liposome formation with methanol. The encapsulation efficiency and morphological appearance were followed with evaluated stability of ketoconazole liposome formulations at 4 °C and 25 °C, during a month. After the first week and one month of the liposome preparation, during stability test at 4 °C, the encapsulation efficiency was 43% and 40%, respectively. At 25 °C, the encapsulation efficiency was decreased to 40% after the first week of the preparation, while after one month, the determined encapsulation efficiency was 45% less than the initial value. The uniformed liposome appearance with a tendency of aggregation at higher temperature (25 °C) during one month stability test was confirmed using microscopic analysis. The drug leakage increases at higher temperature as a result of higher lipid fluidity (Patel et al., 2009).

Conclusion

The injection method using methanol could be applied in the ketoconazole liposome preparation. Process and formulation parameters should be further optimized to obtain vesicles with higher encapsulation efficiency, improving stability after preparation, as well as in simulated conditions, and sterilizing the obtained liposomes.

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Short communication

In vitro model for the analysis of 12-monoketocholate impact on simvastatin physico-chemical behavior in octanol/buffer system

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Introduction

The octanol-water distribution coefficient represents a physico-chemical property of a compound, being a valuable parameter in the understanding of its biological behavior. It has been widely used in numerous quantitative structure-activity relationship (QSAR) models for predicting the pharmaceutical properties of molecules (Hughes, 2008). Determination of distribution coefficient is commonly used to predict the drug transport through biological membranes and to test the effect of drug promoters on drug distribution (fanić, 2016). Bile salts are known for their function as modifiers of drug penetration across the biological membranes (Stojančević, 2013). The effect of bile salts on drug penetration appears to be complex and it depends on the physico-chemical properties of the drug and on interactions of the bile salt with the physiological environment. Taking into account that simvastatin (SV) is a highly lipophilic compound with extremely low water-solubility and bioavailability (<5%), many efforts have been made to increase the aqueous solubility of the drug consequently leading to the increase of its bioavailability. Accordingly, the aim of this study was to estimate the influence of semysinthetic bile salt, sodium 12-monoketocholate (MKC), on the distribution coefficient of simvastatin and to suggest molecular mechanisms responsible for this effect.

Material and methods

Distribution coefficients (logD) of SV with or without MKC were determined using a "flask-shake" method at pH 5 and pH 7.4, resembling gastrointestinal environment. The aqueous media used for these pH conditions were 0.1 M sodium acetate and 0.035 M sodium phosphate buffer, respectively, while octanol was used as organic solvent. Experiments were performed according to the procedure described by Serajuddin et al. (1991). Concentrations of SV were determined by high performance liquid chromatography method according to method described by Carlucci et al. (1991). The detection was performed at 238 nm. In order to analyze theoretically complexation of SV with MKC, semi-empirical PM3 method implemented in MOPAC software package in the Chem3D Ultra 7.0.0 program has been applied. It was also used for computation of physico-chemical properties of observed compounds to give a better interpretation of obtained in vitro results. Data were analyzed using OriginPro Software (OriginLab Corporation, MA, USA).

Results and discussion

As a highly lipophilic molecule and weak acid, in more acidic environment, SV is expected to be more in its neutral form that can more easily penetrate biological barriers which explains higher values of distribution coefficient at lower pH (4.70±0.01 at pH5 vs. 4.59±0.06 at pH7.4). The experimental logD values of SV were in good agreement with the calculated logD values reported by Serajuddin et al. (1991). Upon addition of MKC, the distribution coefficient of simvastatin significantly decreased at both selected pH (4.60±0.02 at pH 5, and 4.41±0.05 at pH 7.4). This means that the concentration of SV, i.e. the solubility of simvastatin, in buffer layer is increased in the presence of bile salts. Value logD in system octanol/buffer is dependent on drug solvent interaction. Chemical structure of bile acids is different from ordinary aliphatic surfactants, due to

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the presence of a large, rigid, and planar hydrophobic moiety of a steroid nucleus carrying 2–4 hydroxyl groups and an ionic head of a carboxyl group, which provide the molecule a planar polarity with hydrophilic and hydrophobic domains (Mikov, 2006).The increase in SV solubility correlates with known high hydrophobicity of SV for which strong hydrophobic interactions with the lipophilic steroid nucleus of bile salt are to be expected and are confirmed in analyzed complex SV-MKC. Significantly higher values of Conolly Accesible Area (SAS), Conolly Molecular Area (MS), and Conolly Solvent Excluded Volume (SEV) of SV-MKC complex than SV alone additionally support experimentally obtained results. MKC has similar structure to cholic acid, differing only in a keto group at the position 12 instead of hydroxyl group. It has been shown that replacing of hydroxyl with keto group in MKC resulting in decreased membrane toxicity without compromising ability to enhance membrane permeability (Lalic-Popovic, 2013; Stojančević, 2013), which makes it as a good candidate for the novel drug formulations.

Conclusion

Our data indicate that the addition of MKC into the octanol/buffer system decreases the values of SV distribution coefficient. This may be the result of the formation of hydrophilic complexes increasing the solubility of SV that could consequently lead to the increase of its bioavailability. Results of this study could contribute to the development of new formulations with improved pharmacokinetic properties and enhanced bioavailability of SV.

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Short communication

Influence of the particle size at oleoresin extraction from red hot pepper

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Introduction

The pepper (*Capsicum annuum L.*) which belongs to the genus Capsicum is a widely cultivated and distributed vegetable crop (Huq and Arshad, 2010). It is consisted of carbohydrates, proteins, carboxylic acids, minerals, vitamins, and other biologically active compounds. The green, yellow, orange, and red pepper colour is dependent on a carotenoid pigment profile. Capsaicin as the main capsaicinoid compound determined the pungency in the red hot pepper (Kumar et al., 2011). Although the pepper is produced seasonally, it is consumed throughout the year in either various specialties or spices and oleoresin extracts (Huq and Arshad, 2010; Kumar et al., 2011). The pepper is not only popular spice, but it could also be used in plant based insecticide formulations (Huq and Arshad, 2010). The *Capsicum annuum L.* could be differed from shape, pungency level, colour, and content of biochemical compound. The colour could be evaluated from surface and extractable colour aspects (Malacara, 2011). The red colour intensity is considered as the main quality attribute of the red pepper and oleoresins, since it influences on both consumer acceptance and commercial value. The surface colour is dependent on pepper species and conditions during growing, storage, and processing (Belovič et al., 2014). Many factors affect pepper colour loss such as photolytic and thermal conditions, as well as the oxidative degradation of carotenoid pigments (Belovič et al., 2014). Several different methods for the pepper colour evaluation, based on the surface colour measurement and profiling of the carotenoids are reported in the literature (Belovič et al., 2014). Surface colour measuring is the method for describing colour changes closest to sensory visual perception (Malacara, 2011). In this work the influence of the particle size at extraction of pericarp, placenta, seed, and stalk of red hot pepper of Macedonian origin was studied by the determination of the extract yield and surface CIE Lab colour values.

Materials and methods

The red hot pepper (*Capsicum annuum L.,* spp. microcarpum longum conoides, convar. Horgoshka) was grown in the locality Markova Cheshma, Prilep, Republic of Macedonia. Dried fruits of the red hot pepper were cut manually longitudinally with a knife. Pericarp was separated from the seed, placenta, and stalk. Analytical grade petroleum ether (40–60 °C) was supplied from Merck (Germany). An analytical balance Mettler Toledo (Switzerland) was used for the sample weighing with 0.1 mg accuracy. The samples were ground using a Retsch ZM1 mill (Germany). The extraction of the red hot pepper (10 g ± 0.1 mg) was carried out with Soxhlet procedure using petroleum ether (40–60 °C). After 420 min extraction, the solvent was removed at 40 °C, 200 mPa using a rotary evaporator (Devarot Elektromedicina, Slovenia). The steps of

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drying, cooling, and weighing were repeated until the difference between two consecutive weights was smaller than 2 mg. The yield of the extract was calculated based on the dry matter weight of the red hot pepper sample used. The extraction procedure was performed in duplicate under the same operating conditions. Visual colour of the samples investigated was evaluated measuring the CIE Lab coordinates L* (lightness–darkness), a* (red−green), b* (yellow−blue), chroma (C), and Hue (h°) using Dr. LANGE spectra colorimeter (Chelmsford, UK). Colour difference (ΔE) was calculated from a*, b*, and L* parameters, using Hunter-Scotfield's equation (Ergüneş and Tarhan, 2006).

Results and discussion

At extraction with petroleum ether (40–60 °C), 6.42% extract yield was obtained from pericarp with 0.25 mm particle size. Increasing the pericarp particle size from 0.5 to 1 mm, the extract quantity decreased, from 4.60 to 2.68%, respectively. The extract yield from red hot pepper placenta with 0.5 mm particle size was 7.05%. The difference in the extract quantity obtained from the placenta with 0.5 mm and 1.0 mm particle size was 1.49%. At seed extraction, the highest quantity of the extract was obtained in comparison to pericarp, placenta, and stalk. The highest extract yield of 22.32% was determined at seed extraction with 0.5 mm particle size. The obtained stalk extract quantity was the lowest, 3.70, 2.98, and 1.98% with particles size of 0.25, 0.5, and 1.0 mm, respectively. The empirical models tested showed a good agreement between experimental and calculated data for the extract yield ($R^2 > 0.90$). The values of colour parameters varied between the samples of pericarp, placenta, seed, and stalk of red hot pepper. According to the L* values, seed samples were characterized by the brightest (72.28), while pericarp samples by the darkest colour (54.26). The determined values of the colour parameter L* for placenta and seed were close to each other (65.59). High values of redness, a* (32.11) and saturation, C (41.94) were measured for the pericarp samples. The estimated value of a*/b* ratio in seed was the lowest. Hue (h°) values were linear correlated ($R^2 = 0.9793$) with the values for the ratio of red (a*) and yellow colour (b*). The values of the L*, a*, b*, and C decreased with increasing the sample particle size ($p < 0.05$). The sample with 0.25 mm particle size was whiter, vivid, and deep red in colour than samples with particle size of 0.5 mm and 1.0 mm. The highest colour difference before and after extraction with petroleum ether (40–60 °C) was determined for the pericarp with 0.25 mm particle size (ΔE = 27.12). Increasing the particle size from 0.5 to 1.0 mm, ΔE for pericarp was decreased from 22.72 to 14.78. The influence of the particle size during extraction of placenta is insignificant. For placenta with 0.5 mm and 1.0 mm particle size, estimated ΔE values were 19.28 and 18.67, respectively. The lowest ΔE values were determined during stalk extraction. The values of colour parameters for obtained extracts from pericarp, placenta, seed, and stalk at extraction with petroleum ether (40–60 °C) correlated with the determined colour values of the samples before and after extraction. The colour of red pepper is controlled by carotenoids. Capsanthin, capsorubin, and xanthophylls are responsible for the red, while β-carotene and zeaxanthin for the yellow-orange colour. The red colour of different spices of pepper is mainly due to the biosynthesis of keto-carotenoids such as capsanthin and capsorubin (Ergüneş and Tarhan, 2006). The published data for the surface colour were related to the pericarp as an edible part of the pepper fruits. The differences in the pepper varieties, the maturity degree of the pepper, as well as the influence of drying and processing conditions were studied using characteristics of the surface colour (Belovič et al., 2014; Zaki et al., 2013). Results for the visual colour samples of pericarp are in agreement with those reported in the literature (Belovič et al., 2014; Ergüneş and Tarhan, 2006; Zaki et al., 2013).

Conclusion

The extraction of pericarp, placenta, seed, and stalk of red hot pepper was carried out with petroleum ether (40–60 °C). The bigger particle size enabled to obtain less extract yield. The highest quantities were extracted from seed, while the lowest quantities from stalk. The colour differences increased with decreasing the particle size. The highest colour differences were estimated at extraction of pericarp.

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Development of nanoemulsion formulations of wild oregano essential oil using low energy methods

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Introduction

Essential oils are aromatic, volatile, natural oily liquids obtained from plants, mainly leaves and flowers, as well as from fruits, seeds, roots, and stems. The most common known essential oils are those from lemongrass, oregano, peppermint, rosemary, thyme, cinnamon, and clove. A variety of volatile compounds such as terpenoids, phenol-derived aromatic and aliphatic compounds were determined in the essential oil composition. The essential oils are recognized for their medicinal value such as analgesic, sedative, anti-inflammatory, spasmolytic, and locally anaesthetic remedies. As a powerful natural products with bactericidal, virucidal, fungicidal, antiparasitical, and insecticidal properties, the essential oils take a significant place in cosmetic, pharmaceutical and food industry, as well as in agriculture (Hüsnü Can Başer and Buchbauer, 2010). Concerning the complex chemical composition, the essential oils could be easily decomposed. Oxidative and photolytic conditions resulted in a quality loss and reducing of the biological activity (Bilia et al., 2014). In order to protect and to use in drug delivery systems, nanoencapsulation of the essential oils was proposed due to their capability of improving the solubility, stability, and efficacy of essential oil-based formulation. Nanoemulsion is dispersion of two immiscible liquids with droplet size ranging from 20 to 200 nm (Solè and Solans, 2013). Low and high energy methods are used in the development of nanoemulsion formulation. The high energy method required the mechanical energy such as an ultrasonic procedure and a high pressure homogenization. On the other hand, surfactants as emulsifiers either in aqueous phase titration or in spontaneous emulsification low energy method are used (Schuh et al., 2014).

The objective of this study was to develop stable nanoemulsions of wild oregano essential oil using low energy emulsification methods.

Materials and methods

Surfactants: Tween 80 (polyoxyethylene sorbitan monooleate, Sigma–Aldrich, Germany), Tween 20 (polyoxyethylene sorbitan monolaurate, Merck, Germany), and Span 80 (sorbitan monooleate, Fluka, Germany) were used. Propylene glycol (PG) was supplied from Aldrich (Germany). Wild oregano essential oil (Origanum minutiflorum) produced in Turkey was purchased from Inter Connection Group DOOEL (Republic of Macedonia). MilliQ PURElab Classic system (ELGA, USA) supplied ultrapure water.. An analytical balance Mettler Toledo (Switzerland) was used for the sample weighing with 0.1 mg accuracy. A magnetic stirrer (type MM–530 Tehnika Železniki, Slovenia) and a vortex (EV–102 Tehnika Železniki, Slovenia) were used in the preparation of the wild oregano na-

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noemulsion formulations. Pseudo ternary phase diagrams consisting of wild oregano essential oil, mix of surfactans Tween 20, Tween 80 and Span 80 (Smix), and water were constructed using aqueous titration method (Shaaban and Edris, 2015). In glass vials, the wild oregano essential oil was mixed with Smix in weight ratios from 1:9 w/w to 9:1 w/w and was titrated with calculated amount of aqueous phase (water or water and PG in the ratio 1:1 w/w). After 24 h the samples were monitored, visually inspected for transparency and followability and water was added. The phases were identified as transparent/translucent emulsion, transparent gel, turbid gel or turbid/milky emulsion. The visually evaluated mixtures and nanoemulsion phase were marked on the phase diagram. The droplet size, polydispersity index (PDI), and ζ-potential were measured with Zetasizer Nano ZS (Malvern Instruments Ltd, United Kingdom) 24 h after the emulsion preparation.

Spontaneous emulsification in three step process method was employed (Schuh et al., 2014). In the first step organic and aqueous phase were prepared. The homogeneous organic phase contained oregano essential oil, lipophilic surfactant Span 80, and ethanol as water miscible solvent, while aqueous phase was composed of water and hydrophilic surfactants, Tween 20 or Tween 80. In the second step organic phase was injected into the aqueous phase by stirring on the magnetic stirrer for 30 min at 750 min^{-1} to reach phase system equilibrium. In the third step, ethanol was evaporated on a BUCHI rotary evaporator system type R-114 and vacuum controller B-721 (Switzerland) at 40 °C, 250 bar, 1 h. The influence of the surfactant mixture (Tween 80 and Span 80 or Tween 20 and Span 80) and the surfactant to oil ratio on the droplet size, polydispersity index (PDI), ζ-potential, the particle size distribution, and average particle diameter were determined 24 h after the emulsion preparation.

Results and discussion

In the phase diagrams constructed using the oil, Tween 80, and a surfactant mixture of Tween 80 and Span 80 in the ratio of 2:1 w/w and 1:1 w/w, the effective surface of formed nanoemulsion was smaller. The maximum amount of water incorporated in the system was 23%. As a result of the small amount of water, as well as the high nanoemulsion density, the preparation of water based nanoemulsion was disabled. Substituting Tween 80 with Tween 20 and changing water with a mixture of water and PG in the ratio of 1:1 w/w, nanoemulsion surface increased. When the oil and the Smix in the ratio of 1:9 w/w were used, the formed nanoemulsion could be fully diluted with water without the formation of turbid-white emulsion. The average droplet size measured 24 h after preparation was 44 nm, while PDI was 0.349. Transparent emulsion was not formed when mixture of Tween and Span 80 in the ratio 1:2 w/w was used.

The droplet size of the nanoemulsion prepared using the spontaneous method influenced by the surfactant mixture (Tween 80 and Span 80 or Tween 20 and Span 80) and the ratio of the mixed surfactants and oil (the weight ratio of Smix:oil was 0.5, 1 and 1.5 w/w). The droplets with 160 nm particle size, 0.097 PDI, and -26 mV ζ-potential was obtained using a mixture of Tween 20 and Span 80 mixed with the oil in ratio of 0.5. Using the surfactant mixture of Tween 80 and Span 80 in the proportion of 0.5 with the oil, the droplets with 180 nm particle size, 0.134 PDI, and -20 mV ζ-potential were obtained. Increasing the Six to oil ratio at 1 and 1.5, nanoemulsions with bigger droplets were obtained. The average droplet size of Tween 20 or Span 80 mixture oil in the proportion of 1.5 was 361 nm, while PDI was 0.071 and a low ζ-potential value was recorded (-2.69 mV).

Conclusion

The low energy methods, aqueous titration and spontaneous emulsification method can be used in order to produce fine droplet nanoemulsions of wild oregano essential oil. Aqueous titration method requires high concentration of surfactants at low oil concentration. Spontaneous emulsification method can only be applied when low oil concentration, less than 1%, is used. In order to improve the emulsification efficiency of the essential oil with high concentration of carvacrol as a biological active compound and to increase the stability of the nanoemulsions preparation using aqueous titration and spontaneous emulsification method, the process and working parameters should be optimized.

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Short communication

Risk assessment in blister packaging

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Introduction

Every product and every process has an associated risk. Every enterprise should have a methodology for identifying and evaluating the risks it faces and it should have a process for generating intervention plans to reduce the risks to an acceptable level. This process is generally referred to as a Quality Risk Management (QRM). QRM is a systematic process for the assessment, control, communication and review of risks to the quality of the medicinal product across the product lifecycle. Early in development, the purpose of the QRM process may be to acquire sufficient product and process knowledge to assess risks associated with formulation development of the finished pharmaceutical product according to the quality target product profile. As development progresses, in addition to supporting that development, the purpose of the QRM process is to determine and manage risks to bioavailability, safety, efficiency and product quality.

Risk assessment is a systematic process of organizing information to support a risk decision to be made within a risk management process. It consists of the identification of hazards and the analysis and evaluation of risks associated with exposure to those hazards.

Packaging represents a critical manufacturing operation requiring strong Good Manufacturing Practices (GMPs) and quality oversight to ensure sustained and robust compliance. Historically, inadequate packaging practices have been a meaningful ongoing contributor to product recall actions industry-wide. A strong understanding of the compliance risks associated with product packaging is a necessary and important component of a good quality system. The goal of risk assessment is to further enhance the quality assurance of existing packaging operations and practices (Guidance for industry "Q9 Quality Risk Managment", 2006) (Quality Risk Management Q9, 2005).

Risk assessment

The aim of risk assessment in blister packaging is to identify, analyse and evaluate risks during blister packaging process validation and the manufacturing processes.

Risk identification is a systemic use of information to identify potential sources of harm referring to the risk or problem. Information can include historical data, theoretical analysis, and the concerns of stakeholders. Risk identification addresses the "What might go wrong?" question and provides the basis for further steps in the quality risk management process (Reddy et al., 2014).

Risk analysis is the estimation of the risk associated with the identified hazards. It is the qualitative or quantitative process of linking the likelihood of occurrence and severity of harms (detectability) is also a factor in the estimation of risk (Reddy et al., 2014).

A team is assembled to collect relevant data about the packaging line. All operations involved with the line function (equipment, procedures, etc.) and all corresponding potential failures are listed. For each potential failure, the team works to understand its potential impact on packaging operations and then respectively to assign a severity category. Following severity classification, the team is reviewing all dominant causes relevant to the defined potential failure and then assigning a frequency category. For each potential failure, all safeguards (e.g. detection capabilities) are reviewed and a detection capability is assigned. For Severity Frequency and Detection determination, all relevant data is taken into consideration, to include maintenance and operation logs, batch records, deviation investigations, customer complaint records, etc. (Reich et al., 2011). The determined parameters and operations are: the environmental conditions during the process of primary packaging, issuing packaging material, issuing bulk product, blister forming, filling tablets into blisters, testing tablet presence in the blister, text printing on the aluminum lidding foil, blister sealing, embossing batch number and expiry

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date on the blisters, the blister perforation, the blister cutting, insertion of proper number of blisters, leaflets folding, pharma code reading on the boxes, pharma code reading on the leaflet, embossing batch number and expiry date on the boxes, leaflet presence, weight check, determination of yield. For all parameters and operations potential risks are defined, consequences (severity of consequences, probability of occurrence, the possibility of detection). The assessment effort requires that potential product defects from any given packaging operation is defined and graded for severity, frequency and on the ability of the operation and/or an operator to detect the defect (Internal procedure: List of evidence for identification of risk, 2009)

Risk evaluation compares the identified and analyzed risk against given risk criteria. The output of a risk assessment is estimated as quantitative description of a range of risk. A numerical probability is used. Defect detection is categorized and graded on a 1-to-4 scale to reflect a detection capability of "none" (unable to detect) to "always" detect.

For each potential failure, all precautions are reviewed and a detection capability is assigned. For determinations of severity, frequency and detection, all relevant data is taken into consideration, including maintenance and operation logs, batch (Internal procedure: List of evidence for identification of risk, 2009).

Conclusion

The risk of blister packaging process is reduced to an acceptable level. According to the results of the risk assessment on blister packaging on the IMA C80/A81 blister packaging line, we can conclude that the blister packaging process is with low risk and the process is capable to perform effectively and gives reproducible products with quality that matches with the regulatory specifications and quality attributes.

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Current therapeutic options and trends in drug development for Alzheimer's disease

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Introduction

Dementia is characterized by persons` significant cognitive decline and interference with everyday life independency. There are several types of dementia, but Alzheimer's disease (AD) is most common form. AD a neurodegenerative disease is characterized by decline in memory, language, problem-solving and other cognitive skills that affects a person's ability to perform everyday activities (Alzheimer's, 2015).

Currently there are only 5 drugs approved for AD treatment and all are targeted towards metabolic disorders associated with the disease. Four of them, tacrine, donepezil (DON), rivastigmine (RIV) and galantamine (approved in 1993, 1996, 1998 and 2001, respectively) are in group of acetylcholinesterase inhibitors (AChEi) and one, memantine (MEM), approved in 2003, is N-methyl-D-aspartate (NMDA) receptor antagonist (Cummings et al., 2014). Although AD drugs are frequently used for more than 20 years there are evidences about controversies related to their efficacy and clinical significance (Lanctôt et al., 2009).

The aim of the current study was to analyze safety and efficacy of dosage forms of AD most commonly used drugs with focus on meta-analysis as well as to identify trends in AD treatment.

Current therapeutic options

Oral solution, hard capsules and transdermal patches with RIV, for treatment of mild to moderate dementia associated with AD and Parkinson's disease are present on the market. Latest Cohran`s collaboration review is addressing RIV clinical efficacy/safety in treatment longer than 12 weeks (any dose and administration route) in patients with AD dementia compared to placebo as well as comparison of RIV patches and capsules given at the manufacturer's recommended dose (Birks and Grimley Evans, 2015). Results from the study indicated that RIV use is associated with some benefits on some outcomes (cognitive function, activities of daily living and clinician rated global impression) and no differences in behavioural symptoms and quality of life of carers compared to placebo. No difference compared to placebo for withdrawals before the end of the treatment and at least one adverse effect was identified for RIV lower dose (1-4 mg/day), while for higher dose (6-12 mg/day) results favoured placebo. Fewer side effects related to nausea, vomiting, dizziness, effect on decreased appetite and asthenia for 9.5 mg/day patches compared to capsules were identified (Birks and Grimley Evans, 2015).

DON for the treatment of mild to moderate AD is approved in form of film coated and dispersible tablets. Effects of DON (any dose) in mild, moderate or severe dementia in AD treatments longer than 1 day compared to placebo were assessed by Birks and Harvey (2006). Results were similar as for RIV (Birks and Grimley Evans, 2015) with the exception of positive findings for DON effect on behaviour. Dose of 5mg/day DON was found to be more cost-effective as 10 mg/day dose was associated with more adverse effects and only marginally higher effects.

Comparison of safety and efficacy of AChEi for AD was done by Birks (2006). Four studies were identified, but only one (DON vs RIV) met criteria for inclusion in meta-analysis. No differences in efficacy were observed, but DON was associated with less adverse effects, particularly nausea, vomiting, anorexia, weight loss, event of fall and hypertension. However, conducted meta-analysis suggested that their tolerability might be match if RIV is carefully and gradually titrated for more than 3 months (Birks,

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2006).

MEM is marketed in form of film-coated tablets for treatment of moderate to severe AD dementia. McShane et al. (2006) meta-analysis was related to efficacy and safety of MEM (any dose and any route of administration) for people with AD, vascular and mixed dementia. Clinically significant reduction of deterioration in patients with moderate to severe AD dementia was determined when MEM in dose of 20 mg/day for at least 28 weeks was compared to placebo. In general, MEM was well tolerated with low incidence of adverse effects (McShane et al., 2006).

Latest FDA approved drug for treatment of AD dementia (in 2014) is Namzaric (memantine hydrochloride extended-release + donepezil hydrochloride).

Trends in Alzheimer's disease drug development

Having in mind that we are facing with lack of efficacious therapeutic options for prevention and treatment of dementia and specifically for AD and associated behavioral and physiological symptoms there is extensive scientific research in this field. For ex. search on Scopus database (www.scopus.com.scopeesprx.elsevier.com) performed on 1.11.2015 using key words "Alzheimer's disease" resulted with 105860 items. Annual analysis pointed to the growing scientific interest for AD research as number of published items continuously increased during the years. First 2 published articles related to AD were found to be in 1912 year and the number was relatively low till 1988 (less than 800 per year). Further on, AD research rapidly grew with more than 4000 published scientific papers in 2005, thus reaching its maximum in 2014 with >8000 published scientific items (at the time of search for 2015 there were 6417, and 60 for 2016 year). Also, search on www.clinicaltrials.gov.mk carried out on 27.10.2015 resulted with total of 1769 AD clinical trials allocated to all continents around the globe. At the time of search 541 clinical trials were with status OPEN, mainly in USA, Europe, China and Japan - 231, 175, 67 and 41 respectively. In Europe most of the studies (56.6%) were located in France, followed by United Kingdom (18.3%) and Spain (17.1%). Nearly 30% of active trials were related to drug substances not yet approved for AD. Beside high number of clinical trials we have to be held in reserve as for a period of 10 years (2002-2012) 244 AD drugs were in clinical trials, but only one, MEM in 2003, was approved (0.4% succeed rate) (Alzheimer's, 2015). It is a fact that development of new drugs for treatment of AD is difficult and complicated due to the high costs, relatively long time needed to observe disease progression in AD and blood-brain barrier. Problems associated with blood-brain barrier might be surmounted by innovative therapeutic systems (ITS) for drug targeting and controlled release (liposomes, micelles, nanoparticles etc). Namely, utilizing their unique physico-chemical and biopharmaceutical properties drug targeted and controlled release dosage form with modified/improved pharmacokinetics/dynamics might be developed. Conducted search on Scopus database (accessed on 13.08.2015) retrieved 666 scientific papers (SP) related to micelles, liposomes, solidlipid nanoparticles, nanostructured lipid carriers and nanoparticles as ITS and/or RIV (197 SP), tacrine (124 SP), MEM (189 SP) and DON (156 SP) as drug substances. However in detail analysis of retrieved items revealed that only 15 (2.25%) were original research papers, and others were reviews. A vast majority of the original research papers (86.7%) were related to RIV and only 6.7% were related to DON and MEM, each. Liposomes and micelles as carriers for RIV were studied in 26.7% and 6.7% of the research papers, respectively. On the other hand most of the conducted research (66.7%) was related to nanoparticles, where's 80% were focused on RIV and 10% on DON and MEM, each. All of them were characterized in terms of particle size, drug content/ encapsulation efficiency, zeta potential and in vitro dissolution and majority (66.7%) conducted in vivo animal studies. All obtained results in the reviewed studies pointed to ITS potential use for AD efficacious treatment.

Based on the retrieved data it can be concluded that although ITS for different conditions are already in use for more than 20 years (1989 FDA approved Diprivan® - surfactant-based nanoformulation of anestetic Propofol) exploration of their potentials for AD treatment is still in its begging's.

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Protein corona evolution on polymer nanoparticles for targeted drug delivery

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Introduction

In the recent years, mutual interactions between nanoparticles and various biomolecules have been increasingly studied, in view of the well-known fact that they have major impact on nanoparticle overall performance. After intravenous administration blood is the first biological environment nanoparticles are exposed to, comprising a pool of several thousand proteins in different concentrations (Monopoli et al., 2011). Plasma proteins tend to adsorb on the naoparticle surface forming so-called protein corona, which governs the nano-bio interactions and determines nanoparticles fate following their administration. Modified biological identity of nanoparticles will affect the physiological response including their circulation time, agglomeration, transport, accumulation, internalization and toxicity.

Nanoparticle characteristics (size, shape, surface charge, surface functional groups and hydrophilicity/hydrophobicity), the nature of physiological environment (blood, intestinal fluid, cytoplasm) and the exposure time play a key role in the process of protein corona formation (Masoud et al., 2013). The rate of adsorption/desorption of different proteins, competitive binding, steric stabilization induced by adsorbed polymers and surfactants as well as the protein composition of body fluids lead to dynamic changes of the protein corona.

In the initial phase, usually the most abundant plasma proteins, such as albumin, are adsorbed at the surface of intravenously administered nanoparticles. In time, albumin can be replaced by proteins with higher binding affinity towards NP surface, since the protein corona composition continuously exchange (Vroman effect). It is worth mentioning that there is a possibility for structural and functional changes of proteins that interact with the nanoparticle surface, which could lead to activation of molecular mechanisms resulting in certain pathogenesis (Nel et al., 2009). Therefore, a detailed protein corona characterization is crucial for biocompatibility assessment of the nanoparticulate drug delivery system.

For this purpose, in this study we conducted comparative protein corona composition analysis of three different polymeric nanoparticles at several time intervals.

Materials and methods

Poly(lactic-co-glycolic acid)-b-Poly(ethylene glycol)-b-Poly(lactic-co-glycolic acid) (PLGA-PEG-PLGA) with two different molecular weights (Mw~148000Da and Mw~22000Da) and Poly(DL-lactide-co-caprolactone) (P(DLLACL copolymer, LA:CL 10:90, Mw 77799 Da) were purchased from AkinaInc, USA. Acrylamide-biacrylamide 37.5:1, 30% solution, Electrophoresis running buffer, Laemmli buffer and Comassie brilliant blue were obtained from Bio-Rad, USA. Tris (hydroxymethyl) aminomethane (TRIS) base, Sodium dodecyl sulphate and Amoniumpersulphate were acquired from Sigma Aldrich, USA, while N,N,N',N'-Tetramethylethylenediamine (TEMED) was purchased from MerckMilipore, Germany. All other chemicals were of analytical grade and were used as received.

Nanoparticle preparation

Three types of nanoparticles (NP1, NP2 and NP3) were prepared by nanoprecipitation method as described before (Dimchevska et al., 2015; Koliqi et al., 2016). Following copolymers were used for this purpose: PLGA-PEO-PLGA (Mw~148000Da), PLGA-PEO-PLGA (Mw~22000Da) and P(DLLACL), respectively.

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Protein corona evaluation

Semi-quantitative protein corona evaluation was performed using SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis. 4% stacking gel and 10% resolving gel were used to estimate the protein corona composition of nanoparticles. At first, all NPs (1mg/mL) were incubated with 50% of human plasma at 37 °C for three different time periods (30min, 1h and 4h) in a water bath at stirring rate of 100 oscillations/min (Haake, Denmark). After the incubation, nanoparticles were pelleted by centrifugation (18000xg, 30 min at 4 °C) and the plasma was discarded. The samples were washed twice with 1.5 mL PBS and the supernatant was removed. 25 μL of Laemmli sample buffer containing 5% β-mercaptoethanol was then added to each sample. Samples were vortexed for 30s and heated at 95 °C for 5min in order to provide desorption and denaturation of adsorbed proteins. NPs were allowed to cool down at RT and then 5μL of each sample were loaded into the stacking gel placed on a vertical system (Mini Protean Cell, BioRad, USA). Gels were run for 60-90 min at 110 mV. After the protein separation the gels were removed, immersed in a 0.1% Coomassie blue solution and left to stand for 16h. The next day, each gel was washed with destaining solution (40% methanol, 10% glacial acetic acid and 50% deionized water) in four cycles. Pictures were acquired on white background using VersaDoc, Bio-Rad, USA. Densitometric analysis of proteins was performed using Quantity One 1-D Analysis Software, Bio-Rad, USA.

Results and discussion

Time-resolved protein evaluation confirmed the dynamic character of protein corona for all types of investigated nanoparticles. The results revealed a presence of an intense band around 66 kDa at each time point for all types of NPs, which corresponds to the molecular weight of albumin. Since albumin is the most abundant plasma protein with modular structural organization, high molecular flexibility and variety of binding sites on its surface, mutual non-specific interactions with the NPs were promoted, especially in the early time points of the experiment.

Further, we have noticed that different binding patterns for different NPs over time were displayed, probably due to their specific surface properties. Also, various composition of protein corona for each type of NPs was observed at the later time periods (1h and 4h) which correspond to protein corona dynamic nature and concomitant association/dissociation of diverse protein molecules during time. The results for longer incubation periods have shown that albumin was progressively replaced by other proteins with higher affinity and increased binding specificity to the NP surfaces, regardless of their plasma concentration. Proteins with molecular weights of 43 kDa, 41 kDa, 32 kDa and 25 kDa have gradually replaced the initially adsorbed albumin (66 kDa) as well as proteins with Mw of 55 kDa and 50 kDa at the surface of NP1 and NP2. Additionally, one characteristic band at 8 0kDa was observed at the later time points for NP2 which could not be observed for NP1. Protein corona composition of NP3 formulation was significantly different compared to NP1 and NP2, probably due to the higher hydrophobicity of the NP core. Specific proteins adsorbed onto the NP3 surface were those with Mw of 28 kDa, 36 kDa, 46 kDa, including one high molecular weight protein (Mw 185 kDa).

Our results correspond to the literature data for protein adsorption upon polymer NP surfaces. Namely, in the early time points of the experiment, albumin and fibrinogen were dominant components of the NP protein corona. Later on, significant differences appeared among the adsorbed imunoglobulins upon the surfaces of various NPs and additionally apolipoproteins came to be a significant portion of the total protein quantity.

Conclusion

Displacement of proteins over time was confirmed for all evaluated types of NPs. It was shown that abundant proteins such as albumin and fibrinogen may initially occupy the NP surface and get subsequently replaced by other proteins having higher binding affinity like apolipoproteins.

Different protein binding patterns lead to altered biological identity of NPs that will affect their *in vivo* behavior including their biodistribution, cell internalization, toxicity and efficacy.

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Short communication

Formulation development and characterization of modified release matrix tablets with water-soluble drug

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Introduction

Prolonged-release oral dosage forms offer more advantages over a conventional dosage form of the same drug like sustained blood levels and better patients compliance, smaller dosage unit or higher dosage per unit and economical to manufacture (Hanna et al., 1987). The active agent can now be delivered and made bioavailable in a sustained and well-controlled manner up to a desired period of time. The matrix system is commonly used for manufacturing prolonged-release dosage forms. Bio-functional polymers have pharmaceutical interest in recent years and are widely used for formulation of modified release dosage forms (Meidan and Khan, 2007). Cellulose ethers are commonly employed as the hydrophilic, swellable and erodible matrix polymers for orally administered types of prolonged-release systems (Ferrero Rodriguez et al., 2000). As water insoluble excipients, ethylcellulose polymers (Ethocel) can effectively control the release of an active ingredient by modifying the size and length of diffusion path. By varying the type and amount of the insoluble excipient ratio and the particle size, a wide variety of release rate patterns can be achieved.

The aim of the present study was to develop prolonged-release matrix tablets for water-soluble drug using hydroxypropylmethylcellulose (Methocel) and Ethocel in order to reduce dosing frequency, to lesser the side effects and to improve patient compliance.

Materials and methods

Materials

Water-soluble compound (API) was used as a model drug (solubility 10.2 mg/ml, pKa 10.26 and 9.12, Mw 473.49 g/mol, BCS Class III). The other excipients were: Methocel K100M (Colorcon, EU), Ethocel 7cPs FP (Colorcon, EU), Kollidon K90 (BASF, Germany), Avicel PH 112 (FMC Biopolymer, USA), Aerosil 200 (Evonik, Germany) and Magnesium stearate (Mg S) (Carsco GE, Italy). All the other chemicals used were of analytical grade.

Preparation of matrix tablets by wet granulation technique

The tablets were prepared by wet granulation technique. The API and other excipients were accurately weighed and mixed in laboratory mixer granulator (Diosna Dierks & Söhne GmbH, Germany). The mass ratio of API:Methocel K100M:Avicel112:Mg S was 7.56:29.04: 25.72:14.52:1 for sample 1 and API:MethocelK100M:Ethocel:Avicel 112:Mg S = 7.56:14.52: 14.52:29.04:1 for sample 2, respectively.

Kollidon K90 (5% sol. in alcohol 99%) was used as granulation fluid. The wet mass was passed through #6 mesh and granules were oven dried until a moisture content of 1-3% was achieved. Dried granules were further passed through #230 mesh. The granules were lubricated and compressed into round shaped (7.0 mm) tablets using 4-station rotary compression machine (Korsch XL 100, Korsch AG, Germany) with compression forces 3.8 kN and 8.3 kN (sample 1a,1b and 2, accordingly).

Each tablet contained 15 mg of API in 150 mg tablet core weight. The tablets were evaluated for appearance, diameter, thickness and hardness.

Characterization of the final blends

Bulk density was determined by the following formula: *Bulk density =Ws/Vs*; where *Ws* is sample weight and *Vs* is the sample volume (Ph. Eur. 8.7).

Tape density is the indirect measurement of flow, mixing and tableting properties of powder. Tapped density was determined by Ph. Eur. 8.7 method: tablet blend was filled in 100 ml graduated cylinder of tap density tester which was operated for fixed number of taps until the powder bed volume has reached

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a minimum, thus was calculated by formula: Tapped density = Weight of mixture/volume of mixture after 100 tapings.

Compressibility index of the final blends was determined by *Carr's compressibility (%) = Df - Do/Df*; where *Df* is the bulk density and *Do* is the tapped density (Ph. Eur. 8.7).

Hausner's ratio or index of flowability was calculated by the formula: *Hausner's ratio=V1/V2*; where *V1* is the volume before taping and *V2* is the volume after taping (Ph. Eur. 8.7).

Angle of repose was determined using funnel method. Tablet blend were poured from funnel, that can be raised vertically until a maximum cone height h was obtained diameter heap D, was measured (Ph. Eur. 8.7). The angle of repose was measured using Granulate flow tester (Erweka GmbH, Germany).

Loss on drying is the loss of mass expressed as per cent m/m (Ph. Eur. 8.7). It was measured by drying the substance to constant mass using Moisture Analyzer (Mettler Toledo Excellence HS 153, US).

Physical characterization of the prepared tablets

10 tablets were used to study the weight variation using electronic balance (Sartorius Secura 224-1CU, Sartorius AG,Germany).

The diameter, thickness and tablet hardness was determined for ten tablets using ErwekaTablet Hardness Tester Type TBH 425 TD (Erweka GmbH, Germany).

In vitro *drug release studies*

Dissolution testing was conducted in 900 ml of phosphate buffer pH 6.8 (USP apparatus I, basket, speed 100 rpm) at 37±0.5 °C. A 5 ml of the dissolution medium was withdrawn at regular tome intervals (after 2, 4, 10 and 12 h). The volume of dissolution medium was adjusted by replacing each 5 ml aliquot withdrawn with 5 ml of fresh dissolution medium. Quantity of released API was determined by previously validated HPLC method.

Results and discussion

The bulk density of sample 1 and sample 2 were 0.272 and 0.360 g/ml respectively, while tapped density values were 0.331 and 0.439 g/ml. Compressibility index of sample 1 and sample 2 were 18 and 19%, while Hausner's ratio values were 1.22 and 1.21 correspondingly, which is an indication of fair flow properties.

Angle of repose of the final blends was 41.2° for sample 1 and 40.3° for sample 2. LOD was found to be 2.40% for sample1 and 2.55% for sample2 indicating that both of the final blends had moisture content up to acceptable limit which was good for compression to make uniform matrices.

All tablets were smooth and elegant in appearance. Tablet weight variations for both samples prepared in this study were found to be less than 1%. Sample 1a had diameter of 7.00 mm; thickness 4.23 mm and hardness 5.01 kP (at compression force 3.8 kN). Sample 1b had diameter of 6.97 mm; thickness 3.37 mm and hardness 14.29 kP (at compression force 8.2 kN). Sample 2 had diameter of 6.97 mm; thickness 3.32 mm and hardness 12.12 kP (at compression force 8.2 kN).

Polymers belonging to hydrophilic matrix systems, when exposed to an aqueous medium, do not disintegrate, but immediately after hydration form a highly viscous gelatinous surface barrier which control the liquid penetration into the center of the matrix system as well as drug release from the dosage form (Talukder et al., 1996).

Sample 1a released 57.63, 71.96 and 100.78% after 2, 4 and 10 h, respectively. In the same investigated period, sample 1b released 50.44, 69.88 and 100.55%, indicating that there is no significant difference in the drug release from the Methocel matrices compresses at different compression forces. It can be implied that the porosity and/or tortuosity of the prepared tablets after their hydration were not affected by an increase in tablet hardness from 3.8 kN and 8.2 kN. Obtained results in this work were in accordance with the literature data (Ravi et al., 2008).

Sample 2 released the 30.75, 50.78, 77.49 and 89.13% after 2, 4, 10 and 12 h, indicating that the fine Ethocel particles contributed to retard the drug release from the matrices. The reduction in release rates with addition of Ethocel was perhaps due to slow hydration of the matrix, based upon the hydrophobic character of Ethocel. The insoluble particles of Ethocel were probably acting as barrier to drug release in the gel layer of Methocel.

Conclusion

Based on these results it can be concluded that the combination of Methocel and Ethocel could be a successful solution in modifying the release of water-soluble drug form matrix system tablets.

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Short communication

Statistical process control as a tool for process understanding and continuous process verification

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Introduction

Statistical process control (SPC) provides a statistical approach for evaluating the process of production and for improving the quality of the process through better understanding. SPC is a process orientated data driven method to improve processes to deliver day after day results that have added value for the customer. In particular this method will help tocontinuously improve processes and reduce variability, describe and control the stability of processes, monitor and prove excellence of processes, alert before any batch is violating specifications, and document process understanding for regulatory agencies (Sven, 2016).

When SPC is effectively implemented within a company, benefits can be derived through a reduced cost of manufacture, improved quality, fewer troubleshooting crises, and improved relationships with customers (Thomas et al., 2006).

In this paper the application of SPC in the process of packaging of tablets in the pharmaceutical industry is shown. As a key process indicator (KPI) for the process of packaging the yield of the final product is observed. As a critical quality attribute an assay of tablets in the final product is observed. For SPC studies, individual values - Control Charts and Moving Ranges-control charts were constructed and Process capability indexes were calculated in order to verify if the process is under control.

Materials and methods

Materials used were as follows: Amlodipin Alkaloid tablets 5 mg (Alkaloid A.D., Skopje, Macedonia), PVC foil 250 μm x 118 mm (Lamp East d.o.o., Serbia), aluminum foil Amlodipin Alkaloid tablets 5 mg (Lamp East d.o.o., Serbia), leaflet Amlodipin tablets 5 mg and 10 mg (Zapisd.o.o., Serbia), Carton boxes Amlodipin Alkaloid tablets 5 mg x30 (Grafolikd.o.o., Serbia).

Equipment used was: blister packaging machine IMA TR100LT (IMA S.p.a. Italy), data printing line Videojet 1210 + Etipack Clear (Videojet Technologies Itc, USA, Etipack, S.p.a., Italy), HPLC Thermo Scientific DIONEX UltiMate 3000 (Thermo Fisher Scientific Inc., USA) STATISTICA 8, StatSoft Inc., USA.

Data for the yield of the blister packaging process were obtained as a ratio of produced number of packages of final product Amlodipin Alkaloid tablets 5 mg x 30 and planned number of packages of each batch, expressed as a percentage.

The content of amlodipine in Amlodipin Alkaloid tablets 5 mg was analyzed according to the Ph. EUR. 8 monograph for Amlodipine besylate with HPLC method (EP 2.2.29).

Control charts were generated by STATISTICA software, after the following steps were performed:

- The data for yield and content of Amlodipine were obtained for all batches of Amlodipin Alkaloid tablets 5 mg x30 that were manufactured in 2015;
- The overall mean and average were calculated;
- Moving ranges were calculated;
- Process standard deviations for the process and moving ranges were calculated;
- Control limits for individual values (UCL and LCL) and moving ranges (UCLMR) were calculated (Bakker et al., 2008).

Process capability indexes can be used as a tool that will show how often the process fits into the specification limits. There are two most common used process capability indexes: Cp and Cpk.

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The Cp-value is the relation of the range of the specification to the range of the process and it doesn't show if the process is within the specification limits.

The Cpk-value is the relation of the range of the specification to the mean value to the range of the process. If the value is below 1.0 there are values outside specification. If the value is exactly 0 half of the values are outside specification. All process should have Cpk>1.33 to be sure that the process is stable and always in the desired specification limits.

For the purpose of the study Cpk indexes for the yield of the packaging process and for the content of amlodipine in the finished product Amlodipin Alkaloid tablets 5 mg x 30 were calculated.

Results and discussion

The new approach of the pharmaceutical industry was developed after the Food and Drugs Administration (FDA) announced initiative in August 2002, Pharmaceutical Current Good Manufacturing Practices (cGMPs) for the 21st Century. The modern approach of the pharmaceutical industry has a basis in good process and product understanding. Quality should be built in each phase of the product lifecycle.

As a result of this approach the use of different tools that will help to gain better knowledge for the process is increased in the last 15 years. These tools can be used in all stages of the lifecycle of the drug product. During the manufacturing of the drug products the most common tools that might help in the understanding of the process are: quality risk management, corrective and preventive action, process capability studies, Six Sigma and control charts.

Individual values control charts and moving ranges control charts should be always evaluated together in order to have a complete picture of the process. After the control charts were generated it was concluded that all of the Western Electric statistical rules were satisfied (Bonnie et al., 1956):

1. One point outside of the control limits (3 σ);
2. Two points out of 3 are more than 2 σ from the centre line;
3. Four points out of 5 are more than 1 σ from the centre line;
4. Eight points on one side of the center line

for both control charts: yield and assay of amlodipine in the finished product Amlodipin Alkaloid tablets 5 mg x 30. It was shown on data obtained from 83 produced batches during 2015, that the process of packaging of Amlodipin Alkaloid tablets 30 x 5 mg is under statistical control and there is no special cause variability. Also the Cpk indexes values showed that the process is always capable to give high yield and the active substance content is always within specified limits.

The values for the yield and assay of amlodipine in the finished product Amlodipin Alkaloid tablets 5 mg x 30, obtained from the last 30 batches were used to calculate the control limits for continual process verification. The packaging process of all batches that have been produced from 01.2016 onwards will be followed by control charts with the new established control limits.

Conclusion

In the pharmaceutical industry SPC can be used for better understanding of the process, to analyze existing results for Annual Product Review and as a tool for continuous process verification. Different kind of data can be analyzed as per example: in-process control data (tablet's weight, tablet's height), quality control data (dissolution, content of active substance, and content of impurities) and process performance data (yield).

It can be said that individual value-control charts, moving ranges-control charts and process capability indexes are very simple but valuable tools for process understanding and for following the quality of the product continuously.

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Effects of PSD and wet granulation properties (concentration of granulation aid, temperature and humidity) on physical stability of ascorbic acid 95% granulate

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Introduction

DC (direct compressible) granules nowadays are one of the most popular and most preferable pharmaceutical forms to formulators and as well as to companies because of their easy handling, less time consuming and cost effectiveness aspects.

Granules are manufactured through a complex multistage processes which takes relatively long times under which the starting materials change their physical characteristics a number of times before they take place in final dosage form.

Ascorbic acid is a chemical substance that is best known by its antioxidant activity and free radical scavenging (Heber, 2007). Ascorbic acid is susceptible to oxidative degradation and, therefore, it requires significant physicochemical and stability considerations in its formulations (Odeniyi and Jaiyeoba, 2007).

The purpose of this study was to investigate the variability in the physical stability properties of ascorbic acid 95% granules induced by wet granulation process of ascorbic acid in order to produce ascorbic acid 95% granules used as product which will take place in further processing of conventional tablets.

Materials and methods

Different batches from ascorbic acid 95% granules containing ascorbic acid (95.0% w/w) and hydroxypropyl methylcellulose (HPMC) type 2910 (50 mPas) (5.0% w/w) were prepared by wet granulation technique. Based on before gained experience and literature reference data, concentration of purified water used as granulation aid, process parameters of drying process and sieves/screen size for dry granulate were varied, in order to evaluate the role and check which of this variables is/are most important from the aspect of colour stability of ascorbic acid 95% granules. All manufactured batches from ascorbic acid 95% granulate were stored in stability testing chambers with defined conditions for temperature (40 ˚C) and humidity (75% RH) in open Petri dishes for 15 days and were analysed on zero, fifth and fifteenth day. Physical characterization of granules was conducted using a range of experimental methods.

All excipients used in the present study are approved for use in the pharmaceutical industry. Ascorbic acid 95% granules were produced by using ascorbic acid (DSM), hydroxypropyl methylcellulose type 2910 – HPMC E 5 LV (Methocel, Colorcon, UK).

All methods were performed according to Good Manufacturing Practices (GMP.)

Process parameters: mixing time, mixing speed and chopper speed of the high shear mixer granulator (Diosna 4L, Diosna, Germany) used during wet granulation process were same for all laboratory trials. Mixing of preblend of ascorbic acid (475.0 g) and HPMC E 5 LV (50 g) - mixing time: 3 min, mixing speed: 250 rpm, chopper speed: 1000 rpm. Wet granulation phase was separated in two phases granulation I/II (granulation time: 3 min /6 min, granulation speed: 150 rpm/200 rpm, chopper speed: 1000 rpm/1000 rpm).

Concentration of water purified used as granulation aid during process of wet granulation was varied in different batches as 3.0% w/w, 3.5% w/w and 4.0% w/w.

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Drying process of granules were made in fluid bed drier (Huttling, Germany), by varying of drying parameter in different batches as inlet air temperature 60 °C, 40 °C and 30 °C. As end point determination for drying phase was used loss on drying (LOD) of granules to be maximum 0.5%. In accordance with the varied drying parameters for inlet air temperature, drying time varied from 7 min to 35 min for the drying process. Variations on drying process parameters were made on ascorbic acid 95% granules produced with 4.0% w/w water purified as granulation aid, because based on before gained experience, 4.0% w/w water purified is determined as optimal concentration of granulation aid for obtaining good granulate with acceptable quality parameters.

Prepared dry granules from all batches were separate in two equal parts and were passed through laboratory sieve equipment (QuadroComil, Quadro, Canada) under different sieve size. First part of granules were sieved through sieve with pore size 0.813 mm and the other part from the sieve with pore size 0.610 mm in order to obtained granules with different particle size distribution (PSD).

All manufactured batches of ascorbic acid 95% granules with all variables described above were placed separately in open Petri dish in stability chambers with defined conditions for temperature (40 °C) and humidity (75% RH) and were stored for fifteen days.

Physical and chemical characterization of granules were analysed on zero, fifth and fifteenth day by various experimental methods: Fourier Transform Infrared spectroscopy (FTIR, Varian 660, Australia), Differential Scanning Calorimetry (DSC, Netzsch 204F1 Phoenix, Germany), appearance, colour– organoleptic examination, bulk density and tapped density (Erweka SVM 102, Erweka, Germany), flow characteristics, angle of repose (°) (Erweka granulate flow tester GTB, Erweka, Germany), loss on drying (Mettler Toledo HG 63, Mettler Toledo, Switzerland) and optical microscopy (Morphologi G3S, Malvern, UK).

Results and discussion

From the results obtained in this study it can be easily seen that it is most challenging scientific task to separate just one parameter as responsible for physical (colour) stability issue of ascorbic acid 95% granules. From the produced batches with different variables it is more than obvious that humidity (concentration of granulation aid 4.0% water purified) and time spent under high humidity (concentration of granulation aid 4.0% water purified and dried with 30 °C inlet air temperature which take about 37 min for drying process) are the most responsible factor for changing the colour of ascorbic acid 95% granules and also PSD of the granules plays huge role in colour stability because it was noticed that granules with bigger granules (their fraction with bigger granule) change their colour more drastically than the granules with smaller fraction.

It is important to note that even we have made physical characterization by using range of experimental techniques with none of them except organoleptic and visual examination, changes can't be detected. On the other side by visual examination it can be easily seen that there is big colour difference among the granules.

Conclusion

Results present in this study indicates that production of ascorbic acid 95% granules due to the nature of active substance is very complex process and as most important factors which affect physical stability of granulate, humidity and temperature can be pointed.

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Short communication

Preparation of doxycycline loaded chitosan microparticles for periodontal disease treatment by TPP ionic cross-linking combined with spray drying

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Introduction

Periodontal disease is simply defined as bacterially induced chronic infectious-inflammatory disease that affects the tissues that support and anchor the teeth. It is a disease very difficult to treat, with very little change in the treatment over the last several decades. So, even though with limited efficacy, mechanical plaque biofilm disruption is still at the mainstream of periodontal therapy with the addition of several treatment modalities (systemic antibiotics, topical antimicrobials, laser therapy etc.) but only as adjunct to scaling and root planning. However, optimism for discovery of the treatments with improved efficacy is restored due to continuous research efforts especially during the last two decades, and revised as well as upgraded understanding of the ethyology, pathogenesis and complexity of this disease. Unquestionably, understanding of the complex microbial community, its virulence and the impact upon the host cells response as well as the importance of the host inflammatory-immune response to intraoral plaque for the disease development and progression creates new perspectives in management of periodontitis. Basically, novel research data clarified that the disease outcome depends upon the host response towards the complex dysbiotic oral microbial community (Yucel, 2015). Additionally, it may be modified by genetic and different environmental factors. Consequently, host modulation therapies are being proposed (Deshmukl et al., 2011) in order to tackle excessive host inflammatory reaction and targeting of various aspects of the inflammation pathways (levels of enzymes, cytokines, prostanoids, as well modulation of osteoclast). Host modulating agents include non-steroidal anti-inflammatory drugs (NSAIDS), sub antimicrobial dose doxycycline as well as topical doxycycline, systemic bisphosphonates, host modulating agents antagonizing pro-inflammatory cytokines, selective inhibitors of nitric oxide synthase, inhibitors targeting the signaling pathways like c-Jun N-terminal kinases (JNK) inhibitor; Extracellular-Signal-Regulated Kinases (ERK) inhibitor; NFκB inhibitor; JK3 inhibitor etc.Tetracycline apart from its antimicrobial property, when used in sub-antimicrobial doses works as anti-inflammatory agent and has capability of inhibiting the activities of neutrophils, osteoclasts, MMP 8, thereby inhibiting tissue and bone destruction. However, systemic therapy and conventional polymer implants with Doxycycline hyclate could not supply sufficient concentration of the active substance at the site of action for a prolonged period of time in order to provide efficient therapy. Smart bioadhesive micro and nano drug delivery systems with controlled release of the active substance will greatly contribute to improved therapeutic efficacy, considering their ability to interact with the permeable junctional epithelium as well as elements of inflammation like macrophages, dendritic cells etc, at the same time allocating high concentration of Doxycycline in the vicinity and/or at the site of the inflammation.

Therefore, the main objective of this study is development and evaluation of mucoadhesive controlled release chitosan microspheres for local treatment of periodontal disease, with a size span from 1-10 μm, loaded with Doxycycline hyclate.

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Materials and methods

Materials

Doxycycline hyclate was obtained form Kunshau Chemical, China, chitosan with degree of deacetylation 75-85%, sodium tripolyphosphate (TPP), sodium citrate, 2-methyl-2-propanol and calcium citrate were purchased from Sigma Aldrich, Germany and tetrabutilammponium hydrogen sulphate was purshased from Merck Millipore, Germany.

Methods

Microparticles were produced by spray drying method (Buchi Mini Spray dryer B-290, Switzerland) with these conditions: inlet temperature 175 ˚C, aspiration 70%, pump flow 2% and air flow of 600 Nl/h. The colloidal solutions were prepared by ionotropic gelation method, with gradual addition of the cross-linking agent solutions (10 ml) to the solutions of polymers (1% chitosan sol.) and active ingredient (50 mg) (magnetic stirrer Variomag, Multipoint HP 15, Germany). Particle size and the swelling index were determined with laser difractometry (Mastersizer 2000, Malvern Instr., Ltd, UK) using cell Hydro 2000S, (Malvern Instr., Ltd, UK). The swelling degree was measured in diluted 1:2 phosphate buffer pH 7.0 (USP) with similar ionic strength of saliva. Drug release rate was followed in a suspension of microparticles in diluted 1:2 phosphate buffer pH 7.0 (USP), placed in closed tubes in horizontal shaker at 37 °C, (Unitronic OR, Selecta, Barcelona, Spain). The concentration of released doxycycline hyclate in different time intervals as well as drug content and efficacy of encapsulation were determined using HPLC method, column Phenomenex® 250-4.6 mm, PolymerX 5µm RP-1 100A (Phenomenex, Torrance, CA, USA), mobile phase of 2-methyl-2-propanol, buffer pH 8.0 R (Ph.Eur.), tetrabutilammponium hydrogen sulphate, and diluted solutions of sodium hydroxide and sodium edetate.

Results and discussion

Doxycycline loaded microparticles were prepared by TPP ionic cross-linking combined with spray drying method. Since the cross-linking of chitosan depends on the availability of the cationic sites and the negatively charged TPP species, the crosslinking process was carried out at pH 4.5, where chitosan as a polycation (pKa 6.3) will present –NH+3 sites and mostly phosphoric ions from TPP will be generated (Desai & Park, 2004). Different concentrations of the TPP cross-linking solutions (formulation A - 0.1% TPP; B - 0.5%; C - 0.75%; D - 1%) were used in order to evaluate their influence upon the physico-chemical properties of the chitosan microparticles like, particle size and particle size distribution, yield, efficacy of incorporation, drug content and dissolution rate.

The results pointed that at higher TPP concentration larger particles were produced with a presence of aggregates after the spray drying process. Mean particle sizes were 5.0 µm; 3.3 µm; 7.5 µm and 50 µm for series A, B, C and D, respectively. Span factor gradually increased with increasing concentrations of TPP solution, confirming the aggregation in series prepared using 0.75% (span = 2.9) and 1% (span = 4.8) TPP cross-linking solution. Yield and efficacy of encapsulation were slightly increased for microparticles prepared using 0.75% and 1% TPP. The dissolution studies showed that although dissolution rate decreased with increasing TPP concentrations, the rate reduction was not significant and it may have resulted from the particle size difference and the differences in the surface area exposed to the dissolution medium (formulation B - Dissolution results at T 1h, 3h, 5h were 40%, 60% and 90 cum.% compared to formulation C Dissolution rate results of 35, 50 and 80 cum.% at T 1h, 3h, 5h, respectively).

Conclusion

The present study investigated the influence of different concentration of the cross-linking agent on the physicochemical properties of the spray dried chitosan microparticles. It was found that the TPP concentration influenced the particle size, particle size distribution, yield, drug loading and efficacy of encapsulation, swelling index as well as the dissolution rate of Doxycyline hyclate. Also, higher concentration of TPP resulted in production of larger particles with very broad particle size distribution and substantial formation of aggregates with increasing concentrations of the cross-linker.

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Short communication

Preformulation studies as initial phase in development of film-coated tablets with BCS class II active component

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Introduction

It may seem obvious to state that a new product should be adequately defined before anyserious product development is undertaken. In many cases, the value of the design phase is often underestimated in the rush to start development and get products to the market quickly.

This can result in much wasted time and valuable resources. Since tablets and capsules account for approximately 70% of pharmaceutical preparations, an investigation into the solid-state properties of candidate drugs is an important task to be undertaken during preformulation (Wells, 1988). Generally speaking, when dealing with high strength solid dosage forms, this formulation will be more susceptible to any drug substance variability. However, other studies are also important since, for example, the same chemical compound can have different crystal structures (polymorphs), external shapes (habits), and hence different flow and compression properties.

Preformulation is the initial phase in the development of pharmaceutical products. Suitable preformulation will inevitably result in obtaining simple and elegant formulation and successful dosage form from a commercial aspect.

The performance of a solid dosage form is dependent on the physicochemical properties of the active ingredient and the excipients.

Preformulation is a critical phase in drug development where the physicochemical profiling of the active pharmaceutical ingredients (APIs) and excipients are determined and prototype formulations are made.

Selection of stable polymorph and solid-state compatibility studies of the compounds proposed for development of new pharmaceutical formulations are essential in the initial stage of formulation in order to identify possible incompatibilities that may affect the stability of the finished product (Gibson, 2004).

A wide variety of methodologies that exist make it possible for the preformulation scientist to effectively study whatever needs.

The aim of this study was to choose the most stabile polymorph as well as to investigate possible solid-state interactions between API of BCS class II and excipients proposed for development of film-coated tablets, based on the changes in the infrared spectra of the both polymorphs and induced changes in the infrared spectra of the stressed binary mixtures compared to the infrared spectra of initial binary mixture, as consequence of possible solid-state chemical reaction. Additionally, differential scanning calorimetry (DSC) and thermogravimetric analysis (TGA) were used to distinguish between the pseudopolymorphs of the studied API.

Materials and methods

Materials used are: anhydrous pseudopolymorph of BCS class II active compound, monohydrate pseudopolymorph of BCS class II active compound, mannitol, dicalcium phosphate, povidone, hydroxypropyl cellulose, low-substituted, sodium stearyl fumarate, cellulose, microcrystalline, partially pre-gelatinazed maize starch and sodium lauryl sulfate.

Fourier Transform Infrared (FTIR) spectroscopy has proved to be suitable technique for these trials. The FT-IR spectra were recorded using ATR method, in the 4000–550 cm^{-1} region, on Varian 660 FT-IR spectrometer (Varian, Australia) (resolution 4 cm^{-1}, 16 scans per spectrum). Attenuated total reflectance (ATR) spectra were obtained by MIRAcleZnSe ATR module (PIKE technologies) with

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low pressure micrometer clamp.

FT-IR spectra of the binary mixtures were recorded initially and then the same samples were placed in the stability chambers under various conditions (40 °C/75% RH, 25 °C/60% RH).

Weighed samples of 2-3 mg of the studied API (monohydrate and anhydrous form) were scanned in aluminum pans with a perforated lid at speed of 10 K/min from room temperature to 260 °C. All of the samples were analyzed in dry nitrogen atmosphere using a Netzsch DSC 204F1 Phoenix instrument (Netzsch, Germany). The TGA curves were recorded in the 25–400 °C range, on a Netzsch TG 209 F1 Iris analyzer (Netzsch, Germany) using aluminum oxide pans.

Results and discussion

Results showed spontaneous transition of API from anhydrous to monohydrate form. Significant difference between these pseudopolymorphs was shown in data obtained by Differential Scanning Calorimetry and Thermogravimetric analysis. In the TG curves of monohydrate and anhydrous forms losses of mass of 5.11% and 1.27%, respectively was observed which is in accordance with theoretical value for monohydrate and anhydrous form. The melting endothermic peak at around 253 °C (with decomposition) in the both DSC curves, and continuous evaporation of water in the DSC curve of the monohydrate form was observed. These observations are in good agreement with the studied pseudopolymorphic forms. Stability of the molecule of the monohydratepseudopolymorph was the main reason this polymorph to be chosen as more suitable for the development of film-coated tablet formulation.

Careful inspection of the obtained spectra of the pure API (fresh and stressed) and fresh and stressed binary mixtures, leads to conclusion that no changes in the position and shape of bands in regards to the spectrum of the initial sample are observable. This confirms that the applied stress conditions do not affect the overall appearance of the FT-IR spectra of the API. Therefore, the characteristic vibrational bands from API as well, can be used as relevant spectroscopic markers in order to assess the solid-state stability between APIs in the presence of the studied excipients. Based on the comparison of the FT-IR spectra of the initial and stressed binary mixtures it can be concluded that after exposure on accelerated temperature and humidity levels during the screening period, there is no significant change in the FT-IR spectra of the binary mixtures of API and described excipients in comparison to the corresponding FT-IR spectra of the freshly prepared samples.

Minor spectral changes, in the binary mixture with povidone stored at higher temperature/moisture levels were observed at 1660 cm^{-1} probably due to the higher presence of moisture and hygroscopic nature of the excipients. For this purpose binary mixture with povidone in formulation ratio was prepared and tested by the same procedure as previously described. In this binary mixture there were no significant changes in the FT-IR spectra.

Conclusion

Results showed spontaneous transition of API from anhydrous to monohydrate form. Stability of the molecule of the monohydrate pseudopolymorph was the main reason this polymorph to be chosen as more suitable for the development of film-coated tablet formulation.

The dedicated FT-IR spectroscopy of the binary mixtures between API and the proposed excipients clearly demonstrated the solid-state compatibility of the API and the described excipients. The conclusion was derived based on the absence of significant changes in the FT-IR spectra of the stressed binary mixtures in comparison with the corresponding data obtained from the freshly prepared binary mixtures and the starting materials.

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Influence of the formulation factors on the dissolution of highly dose water soluble active pharmaceutical ingredient

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Introduction

Highly dose water soluble active pharmaceutical ingredient (API) with analgesic effect was formulated as an immediate-release film-coated tablet. This formulation is indicated for treatment of mild to moderate pain and to express the analgesic effect faster.

It is important to note that the film is not functional, and the presented data are related to the tablet core.The purpose of our study was to examine the influence of some formulation factors, such as particle size of the API, disintegrant type and concentration (Bolhuiset al., 1994), mass of tablet core and concentration of starch (starch 1500/ partially pre-gelatinized Maize starch) (Cunningham, 1999) on the dissolution profile of the API.

Materials and methods

API, Prosolv HD 90(Silicified microcrystalline cellulose) was supplied from JRS Pharma Rosenberg, Germany, Starch 1500 (partially pre-gelatinized Maize starch) was supplied from Colorcon, Indianapolis USA, Kollidon VA 64 (Copovidone) was supplied from BASF, Ludwigshaften, Germany, Primojel (Sodium starch glycolate) was supplied from DFE Pharma, Goch, Germany, L-Hydroxypropyl cellulose LH-11 was supplied from Shin-Etsu, Tokyo, Japan, Ac-Di-Sol (Croscarmellose sodium) was supplied from FMC Bio Polymer, Wallingstown, Little Island, Cork , Ireland, Aerosil 200 (Silica, colloidal anhydrous) was supplied from Evonik Industries , Rheinfelden, Germany, Kolliphor SLS Fine (Sodium Lauryl Sulphate) was supplied from BASF, Ludwigshaften, Germany, Magnesium stearate was supplied from FACI SpA, Carasco, Italy and Talc from Merck, Darmstadt.

API used in the experimental trials is characterized with high water solubility and has molecular weight 352.48.

Formulations with different disintegrants, partially pre-gelatinized Maize starch (Starch 1500) concentration, different tablet core mass and API's particle size were prepared:

- Formulation I: API is not sieved, has not Starch 1500/ partially pre-gelatinized Maize starch, with mass of tablet core 875 mg and 5% pro tablet (Rowe et al., 2013) of L-hydroxypropyl cellulose.
- Formulation II: API is not sieved, with 5% pro tablet Starch 1500/ partially pre-gelatinized Maize starch, mass of tablet core 875 mg and 5% pro tablet of L-hydroxypropyl cellulose.
- Formulation III: API is not sieved, with 10.83 % Starch 1500/ partially pre-gelatinized Maize starch, mass of tablet core 875 mg and 5% pro tablet of L-hydroxypropyl cellulose.
- Formulation IV: API is not sieved, has not Starch 1500/ partially pre-gelatinized Maize starch, mass of tablet core 875 mg and 10% pro tablet of L-hydroxypropyl cellulose.
- Formulation V: API is sieved through 610 µm sieve, with 11.83 % pro tablet Starch 1500/partially pre-gelatinized Maize starch, mass of tablet core 875 mg and 5% pro tablet of L-hydroxypropyl cellulose.
- Formulation VI: API is sieved through 610 µm sieve, with 6.33 % pro tablet Starch 1500/partially pre-gelatinized Maize starch, mass of tablet core 875 mg, 5% pro tablet of L-hydroxypropyl cellulose and 5% pro tablet Ac_Di-Sol/croscarmellose sodium.
- Formulation VII: API is sieved through 610 µm sieve, with 5.67 % pro tablet Starch 1500, mass of

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tablet core 860 mg and 4% pro tablet of Primojel/ sodium starch glycolate.

- Formulation VIII: API is sieved through 610 μm sieve, with 10.42 % pro tablet Starch 1500/ partially pre-gelatinized Maize starch, mass of tablet core 865 mg and 5% pro tablet of Primojel/sodium starch glycolate.
- Formulation IX: API is sieved through 315 μm sieve, with 5.67 % pro tablet Starch 1500/partially pre-gelatinized Maize starch, mass of tablet core 860 mg and 4% pro tablet of Primojel/sodium starch glycolate.
- Formulation X: API is sieved through 315 μm sieve, with 10.42 % pro tablet Starch 1500/partially pre-gelatinized Maize starch, mass of tablet core 865 mg and 5% pro tablet of Primojel/sodium starch glycolate.
- Formulation XI: API is sieved through 610 μm sieve, with 8.00 % pro tablet Starch 1500/sodium starch glycolate, mass of tablet core 880 mg and 4% pro tablet of Primojel/sodium starch glycolate.
- Formulation XII: API is sieved through 610 μm sieve, with 8.00 % pro tablet Starch 1500/partially pre-gelatinized Maize starch, mass of tablet core 865 mg and 5% pro tablet of L-hydroxypropyl cellulose.
- Formulation XIII: API is sieved through 610 μm sieve, with 8.00 % pro tablet Starch 1500/partially pre-gelatinized Maize starch, mass of tablet core 880 mg, 3% pro tablet of L-hydroxypropyl cellulose and 3% pro tablet of Primojel/sodium starch glycolate.

Dissolution profile is being conducted on the experimental trials.

The dissolution method conditions were: apparatus II (paddle apparatus) at speed of 75 rpm, dissolution medium (900 ml ± 1% phosphate buffer pH 7.2 ± 0.05), 10 ml sample volume, dissolution medium temperature 37 ± 0.5 ◦C. Samples were collected at six time points: 5, 10, 15, 20, 30 and 45 minutes.

For determination of dissolution rate of active substance UV absorptionspectrophotometric method was used.The content of dissolved API was calculated by measuring the UV absorptions of the test and standard solution at detection of 221 nm.

Results and discussion

Based on the results from the extensive dissolution evaluation on the experimental trials one may conclude that the most influential factor among the following: disintegrant type, tablet average mass and API particle size is the disintegrant type. Namely, the dissolution profile is most highly affected when pre-gelatinized starch is incorporated into the formulation in comparison to sodium starch glycolate and crosscarmelose sodium. This results point to conclusion that dissolution of the highly dosage highly water-soluble API is rather fastened by the partitioning of the dissolution media between the pre-gelatinized starch and the API than with the use of a classical super-disintegrants. The high percent of the API in the formulation support this assumption having in mind that the mechanism of the super-disintegrants is hindered by this high percent of the API (85w/w% of the API in tablet formulation).

As per the second factor, increase of the average mass of the tablet with other excipients (fillers) did not cause any increase on the dissolution of the API due to the fact that this slight percent of mass increase was not enough to modify and support the mechanism of disintegration related to super-desintegrants.

The third factor, particle size of the API (achieved by sizing with different sieve size) was also found to be not influential as the disintegrant type probably due to the API is characterized with high water solubility and penetration of the dissolution media is not hindered by different particle size of the API.

Conclusion

The presented study shows that optimized concentration of Starch 1500/ partially pre-geletanized Maize starch and the combination of two selected disintegrants (L-HPC and Primojel/sodium starch glycolate), as well as sizing step of the active pharmaceutical ingredient have influence on the dissolution rate of the highly dose water soluble API.

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Trastuzumab and its radioimmunoconjugates in treatment of cancer

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Introduction

Monoclonal antibodies are new type of targeted anticancer therapy, which achieve specificity, selectivity and localization in tumor cells. There are many naked antibodies and immunoconjugates commercially approved for different types of cancer (Mehren et al., 2003). In order to improve specificity and selectivity of cytotoxic drugs and toxins, monoclonal antibodies are used for formulation of immunoconjugates. Many efforts are done to develop stable immunoconjugates of trastuzumab with various drugs, toxins and radioisotopes to improve the general conditions of the patients (Sharkey and Goldenberg, 2006). The aim of this paper is to focus on current achievements in the formulation of radioimmunoconjugates of HER2-targeting trastuzumab.

Trastuzumab is a humanized IgG1 monoclonal antibody active against HER2 positive breast cancer. It originates from murine antibody 4D5 that is potent inhibitor of HER2 positive cancer cells. Subsequently, it was chosen for further clinical development in order to reduce the probability of generation of HAMA (human anti-murine antibody) (Harries and Smith, 2002). Carter et al. (1992) cloned hypervariable regions from 4D5 in plasmids which encode formation of constant regions from human IgG1 antibody and generated a vector that encode formation of chimeric antibody which is additionally humanized. The new humanized 4D5 has higher affinity for the HER2/neu antigen and reduced immunogenicity. Trastuzumab is acting by binding to the IV subdomain of the HER2 receptor and Fc region of the antibody support ADCC (antibody-dependent cellular cytotoxicity) (Gennari et al., 2004).

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Radioimmunoconjugates for imaging and therapy

Because of the easy detection, radioimmunoconjugates can be used for body imaging at a molecular level using sensitive imager like γ camera, computed tomography and positron emission tomography (PET) (Goldenberg, 1997). Significant radiopharmaceuticals based on peptide and antibody for diagnostic and therapeutic purpose use different radioisotopes (99mTc/188Re, 67Ga, 177Lu, 90Y, 131I) (Kassis, 2008). In order to obtain successful labeling, previously conjugaton with a bifuntional chelators (DOTA - 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid; DTPA - diethylenetriaminepentaacetic acid; TCMC - (1,4,7,10-tetra-(2-carbamoyl methyl)-cyclododecane; HYNIC - succinimidyl-6-hydrazino-nicotinamide; 1B4M-DTPA - 2-(4-isothiocyanatobenzyl)-6-methyl-diethylene-triaminepentaacetic acid) is required. These chelators allow binding to the antibody on the one side, and coordinative binding of radioisotopes on the other side (Kang et al., 2012).

Immunoconjugates of trastuzumab for PET imaging

In recent years there have been significant achievements in development of stable immunoconjugates of trastuzumab for PET imaging of HER2 positive lesions (Hooge et al., 2004). Chen et al. (2008) used 99mTc in order to create a stable conjugate, 99mTc-NYCIN-trastuzumab, useful for identification of HER2 positive metastasis. Tamura et al. (2010) have shown the possibility of identification of HER2 positive lesions in patients with pri-

mary metastatic breast cancer with 64Ga-DOTA-trastuzumab. Three years later, Alitezapour et al. (2013) were able to formulate similar conjugate with another gamma emitter 67Ga-DOTA-trastuzumab for the same purpose. Investigations of Palm et al. (2003) for pharmacokinetics of trastuzumab labeled with pure β emitters 86Y and 90Y in mice with ovarian cancershown a selective uptake of the conjugate by the tumor cells and minimal localization in healthy organs. In vitro and in vivo investigations in mice with breast tumor show that 177Lu-DOTA-trastuzumab can be new promising drug in treatment of human breast cancer (Rasaneh et al., 2012). Tan et al. (2012) have shown that 212Pb-TCMC-trastuzumab has a significant therapeutic effect in HER2/neu positive prostate cancer. Borchardt et al. (2003) have tested therapeutic effects of alpha emitters 227Th-DOTA-p-benzyl-trastuzumab and 225Ac-trastuzumab in mice with HER2 positive breast and ovarian cancer. Studies have shown rapid internalization and cytotoxicity in cancer cells which leads to a extend survival and low toxicity.

Our examinations will be focused on synthesis and evaluation of the immunoconjugates of trastuzumab with bifunctional chelators (DOTA, DTPA and 1B4M-DTPA) with already used method for freeze dried kit formulation of rituximab-conjugates. The most stable immunoconjugate will be labeled with gamma emitter Ga-68 for further in vitro characterization and in vivo biodistribution. The simplicity of Ga-68 labeling will increase the access of radioimmunoconjugates in hospitals for PET imaging of HER2 positive metastasis.

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Short communication

Trends in radiopharmacy in developing african countries

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Background

This article describes trends in Radiopharmacy in the developing countries and the current status of Radiopharmacy Practice in Eastern Africa.

The main goal of our presentation is to show and to stress the importance of existing problems related to the missing of the exact information on the number, status and size of Radiopharmacy units in African countries as the regional status as well as for the human resources, education, suitable training and local demand for the Radiopharmacy and Nuclear Medicine services is not documented (Dondi, 2006).

The Radiopharmacy Practice by the definition requires well-defined and controlled conditions to avoid any risk contamination with microbes, pyrogens and particulate matter as well as cross contamination with other radiopharmaceuticals, together with established radiation protection.

Implementation of the Good Radiopharmaceutical Practices in all levels in the Radiopharmacy should be planned, introduced by the planned priority and strictly monitored and reordered in the production, preparation, testing and in the packaging areas for all final product ready for use.

The practice of nuclear medicine using established radiopharmaceuticals, mostly from the first generation has clinical applications in virtually all systems of the body, for example, the skeletal, cardiac, endocrine, oncologic, gastrointestinal and renal systems. The commoner nuclear medicine procedures in African developing countries are the bone scan, thyroid scan and the renal scan respectively.Almost all radiopharmaceuticals are parenterally administered and requires techniques and procedures that guarantee sterility of the product done according to the clearly defined protocol and and established and controlled conditions.

The critical moment for the realization of all these necessities is to have suitably staff, enough educated and trained to provide the implementation and development in the right direction.

Radiopharmacy professionals should have an adequate training in all aspects of the sterile production, quality control, GMP, GLP, radiation safety and radiochemistry to ensure the competent handling of the radioactive materials (IAEA NUMDAB, 2009).

Methodology

The practice of radiopharmacy combines the expertise of pharmaceutical preparation and the skills needed to handle radioactive substances. Diagnostic radiopharmaceuticals do not normally have any pharmacologicale ffect and their administration is not associated with major clinical side effects. Their clinical use, however, is associated with a risk deriving from radiation exposure and possible contamination during radiopharmaceutical formulation by chemical, biological and microbiological impurities.

Accordingly, principles of Good Practices should be planned in all levels, by priority and strictly observed in the production, preparation, testing and the packaging of the final product ready for use. The main powerful key for implementing Good Radiopharmaceutical Practice is qualified and trained personnel. Trained and competent staffs are essential for achieving high standards and growth in Radiopharmacy. In African Countries there is an acute shortage and in some countries an absence of nationally registered pharmacists with radiopharmacy experience. Most nuclear medicine facilities operate their radiopharmacies with the support of technologists not trained in radiopharmacy practices. For that reason the basic quali-

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ty systems in the Radiopharmacy laboratory could be improved significantly with the better training.

This competency-based training is the first step to provide the essentials of a trainingprogramme for all radiopharmacy practitioners that addresses the following requirements:

- Standardization of training for staff members that operate in hospital radiopharmacy practice;
- To improve performance and management of the radiopharmacy service;
- To encourage good radiopharmacy practices for the preparation and quality assurance;
- To establish a quality management system which encourages continuous update of core competencies in hot laboratory staff.
- Encourages continuous update of core competencies in hot laboratory staff.

Results

One of the key bottlenecks for Nuclear Medicine is a human resources shortfall, especially radiopharmacists. There is an acute shortage and in many countries absences of pharmacists with radiopharmacy experience. Most of Nuclear Medicine facilities operate at IAEA operational level 1 and 2' mainly with support of technologists. There is a global need for effective implementation of the 'operational guidance on hospital radiopharmacy-a safe and effective approach' under which there is a strong recommendation to strengthen skills, competencies and professional qualifications of all staff involved in clinical radiopharmacy practice. They should be empowered to address the poor state of Radiopharmaceutical laboratories in many countries and be more aware of cost of radiopharmaceuticals.

For safety of patient they should be aware of proper registration of radiopharmaceuticals and quality assessment required locally. At the time when there are difficulties with supply and relative high cost of routine radiopharmaceuticals, trained staffin Radiopharmaceutical laboratories could make the difference to Nuclear Medicine (IAEA NUMDAB, 2009).

The trained radiopharmacist should have a:

1. working knowledge of the radiopharmaceutical terms, abbreviations, and symbols commonly used in prescribing, compounding and dispensing radiopharmaceuticals
2. working knowledge of the procedures and techniques relating to aseptic compounding and parenteral admixture operations.
3. working knowledge of the procedures and operations relating to the reconstitution, packaging and labeling of radiopharmaceuticals
4. the ability to perform the usual functions associated with a specific radiopharmacy.
5. the ability to perform the manipulative and record keeping functions associated with the compounding and dispensing of radiopharmaceuticals
6. manipulative and record keeping function sassociated with quality control testing of radiopharmaceuticals
7. working knowledge of drug dosage by imagingprocedure, routes of administration, dosage forms, and be able to distinguish
8. the ability to perform the essential functions relating to drugpurchasing and inventory control
9. appropriate knowledge and understanding of the specific nuclearpharmacy site with emphasis on the technician duties and responsibilities, including standards of ethics governing pharmacy practice therapeutic from diagnostic radiopharmaceutical utilization
10. ability to perform them a thematical calculations required for the usual dosage determinations and solution preparations in the compounding and dispensing of radiopharmaceuticals.
11. Demonstrate appropriate working knowledge of any additionaltraining or safety requirements mandated by the pharmacy or by any local, state, or federal agency by successful completion of any required program (DAT on-line, 2011).

Conclusion

This paper present our idea how to create suitable training and network of all professionals and state authorities for establishing and develop education for Good Radiopharmacy Practice, qualified personnel and appropriate regulation according to the local and international parameters that will be step forward to have advanced health care system and confidence of the patients.

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Short communication

Solid-state compatibility screening of $CaCO_3$ and $MgCO_3$ with selection of excipients suitable for development of solid-dosage formulation

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Introduction

Fourier transform infrared spectroscopy (FT-IR) was applied as suitable screening analytical method to assess the compatibility screening of $CaCO_3$ and $MgCO_3$ with several excipients aimed for development of solid-dosage formulation.

The solid-state compatibility testing of the active pharmaceutical ingredients (APIs) $CaCO_3$ and $MgCO_3$ and the selected excipients was performed by Fourier Transform Infrared (FT-IR) spectroscopy. The compatibility was studied by comparison of the FT-IR spectra of the pure samples (APIs and excipients) and freshly prepared binary mixture with the FT-IR spectra of the corresponding stressed binary mixtures. The appearance of new bands in the FT-IR spectrum, non-typical for the APIs or excipients, can be considered as most significant sign of possible solid-state interaction and formation of new molecular species. Changes due the water/moisture intake, resulting in band broadening or changes in the band resolution and intensities in the FT-IR spectra, can be considered normal, because the samples were exposed directly to the external influence, without any protection or packaging.

The aim of this experiment was to investigate possible solid state interaction between APIs and the described excipients, based on the induced changes in the infrared spectra of the binary mixtures exposed at different external conditions, compared to the infrared spectra of the pure compounds and the freshly prepared mixtures. The obtained results afforded deeper insight into the solid-state stability of the studied binary mixture.

Materials and methods

During development of the $CaCO_3$ and $MgCO_3$ tablets several excipients were evaluated for compatibility with the active ingredients as a screening for potential choice for tablet formulation. APIs: Calcium carbonate ($CaCO_3$, purchased from Solvay Osterreich GmbH, Austria) and Magnesium carbonate heavy ($MgCO_3$, purchased from Dr.PaulLohmann, Germany); Excipients: Silica, colloidal anhydrous (purchased from Evonik Resource Efficiency GmbH, Germany); partially pre-gelatinized maize starch (purchased from Colorcon, USA); copovidone (purchased from BASF (BTC Europe GmbH), Germany); xylitol (purchased from Roquette, France); low-substituted hydroxypropylcellulose (purchased from ShinEtsu, Japan); spearmint flavor SD (purchased from Symrise AG, Germany); menthol L flavourspraydried (purchased from Symrise AG, Germany); talc (purchased from Merck, Germany) and Mg stearate (purchased from FACI SpA, Italy).

Binary mixtures were prepared by dry mixing of equal amounts of APIs and each excipient in 1:1 ratio (*w*/*w*). This ratio is different than the ratio used in the formulation, but according to the literature (Cunha-Filho et al., 2007), the equal masses afford bigger possibility for solid-state interaction among the constituents of the mixture. Pure APIs, excipients, and corresponding binary mixtures were stressed by 30 days exposure in stability testing chambers, at open Petri dishes, at temperatures of 40 ºC and 25 ºC, with a relative humidity (RH) of 75% and 60%, respectively.

FT-IR spectra of pure APIs and excipients, freshly prepared binary mixture and stressed binary mixtures after 30 days were recorded and evaluated.

The FT-IR spectra were recorded using ATR method, in the 4000–550 cm^{-1} region, on Varian 660 FT-IR spec-

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trometer (Varian, Australia) (resolution 4 cm^{-1}, 16 scans per spectrum). Attenuated total reflectance (ATR) spectra were obtained by MIRAcleZnSe ATR module (PIKE technologies) with low pressure micrometer clamp.

Results and discussion

Based on the comparison of the FT-IR spectra of pure $CaCO_3$ and pure $MgCO_3$, with fresh prepared binary mixtures and corresponding excipients, it can be concluded that in all cases the most prominent vibrational bands of the APIs can be identified in the initial prepared binary mixtures. Although, generally being obscured and overlapped by strong bands of the APIs, few stronger bands originating from the present excipient can be identified in some of the binary mixture. No additional bands, of unknown origin, were observed.

In regards of the FT-IR spectra of the starting binary mixture and the FT-IR spectra of the same mixture after exposure for 30 days at 25 ºC/60% RH and 40 ºC/75% RH, it can be concluded that no significant spectral changes were observed, except in binary mixtures between studied APIs with xylitol and copovidone. In the FT-IR spectra of these binary mixture exposed at stressed conditions some spectral changes were observed according to higher moisture. Having in mind that both of excipients are hygroscopic at high moisture (Raymond et al., 2009), pure xylitol and copovidone were exposed 30 days at 40 ºC/75% RH. Based on the obtained FT-IR spectra, it can be concluded that spectral changes observed in the FT-IR spectra of the stressed binary mixtures are results from the absorbed moisture from xylitol and copovidone, which can be confirmed by their FT-IR spectra.

Conclusion

The closer inspection of the FT-IR spectra of the obtained binary mixtures of $CaCO_3$ and $MgCO_3$ with the proposed excipients, clearly demonstrated that there are no significant spectral changes induced in the FT-IR spectra during the screening period at all testing conditions. The preformulation studies have shown that in the FT-IR spectra in the binary mixtures between: $CaCO_3$ with xylitol and $CaCO_3$ with copovidone; and $MgCO_3$ with xylitol and $MgCO_3$ with copovidone; exposed at stressed conditions, some spectral changes were observed due to moisture uptake by the excipient with hygroscopic properties.

These findings were confirmed by comparison of the spectral changes observed in the FT-IR spectra of the stressed binary mixtures and the individually stressed xylitol and copovidone.

The compatibility studies as a part of preformulation screening of the excipients aimed for development of $CaCO_3$ and $MgCO_3$ solid dosage form were beneficial and give useful directions for development of a stable and effective formulation.

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Short communication

Formulation development of immediate release tablets with water insoluble drug using fluid-bed granulation

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Introduction

Tablets as solid preparations can range from relatively simple immediate release formulations to complex modified release drug dosage forms. The desired drug release properties could be adjusted by proper selection of excipients used in the formulation as well as by technological process and process conditions selected.

Numerous unit processes are involved in making tablets, including blending, granulating, drying, compaction and coating. Various factors deriving from these processes can affect content uniformity, drug release rate and/or stability of tablets.

The fluid-bed method of wet granulation is well known in the pharmaceutical and other industries as a one-step, enclosed operation. Because several ingredients can be mixed, granulated, and dried in the same vessel, the technique reduces material handling and shortens process times compared with other wet granulation techniques. In addition to granulation for tableting, the fluid-bed top-spray method produces highly dispersible granules with a characteristic porous structure that enhances wettability and improves many of the powder properties for tablet compression. Granules of high quality can be obtained by understanding and controlling the critical process parameters by timely measurements (Parikh, 1997).

The aim of this study was to develop immediate release tablets with water insoluble drug (API), using fluid-bed granulation technique.

Materials and methods

Materials

API from BCS Class II/IV (particle size D_{50} less than 20μm, freely soluble in dichloromethane, sparingly soluble in toluene, insoluble in methanol and water) as model drug substance was used. Other excipients used in the formulation were: lactose monohydrate, microcrystalline cellulose, starch maize, hydroxypropylcellulose, croscarmellose sodium, magnesium stearate and iron oxide. All chemical used in this present study are approved for use in pharmaceutical industry.

Preparation of the granules

Three different formulations of granules were prepared using fluid-bed granulation process (Hüttlin GmbH, Hohe-Flum-Strasse 42, Schopfheim, top spray configuration, Germany). Water solution of hydroxypropylcellulose was used as granulation vehicle. Fluid-bed granulation was performed under the following working conditions: air volume 15 m^3/h; inlet temperature 66 °C; atomizing pressure 0.5-1 bar; mycroclimate 0.3-0.8 bar; filter pressure 0.8-1 bar; filter cleaning period 2 sec.; filter blow time 0.2 sec. After finishing the atomization of the binder solution, the granules were dried for a variable period of time in the same apparatus at 66 °C inlet temperature and air volume 15 m^3/h.

For preparation of sample 1, API was granulated together with lactose monohydrate, while starch maize, microcrystalline cellulose and iron oxide were added extragranularly. For sample 2, API was granulated together with all excipients used in the formulation (lactose monohy-

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drate, starch maize, microcrystalline cellulose and iron oxide). For sample 3, API was granulated together with lactose, part of croscarmelose sodium and starch maize, while microcrystalline cellulose, iron oxide and the other part of croscarmelose sodium were added extragranulary.

All prepared samples were finally mixed with magnesium stearate (5 min.; Drum blender, Erweka PM5, Germany) and compressed by a rotary tablet press (KorschXL 100 Pro, Korsch AG, Germany), using round shaped 6 mm punches.

Determination of granules and tablets characteristics

Final blends were evaluated for loss on drying and flow properties. Loss on drying (LOD) was analyzed in Sartorius Infrared dryer MA 35 (Germany). Granulate in quantity of 1 g was dried to constant mass. The loss of mass was presented as percent m/m (Ph. Eur 8.7).

Flowability of granulates was determined according to Ph. Eur 8.7 with Granulate flow tester (Erweka type GTB, Erweka, Germany). Nearly 50 g of granulate was poured from funnel and the time required to empty the funnel was measured.

Determination of disintegration time and dissolution test of the compressed tablets was performed according to the official methods from the European Pharmacopoeia. For that purpose 6 tablets were placed in baskets of apparatus for disintegration (Erweka type ZT302, Erweka, Germany). Time required for disintegration was measured automatically.

Dissolution testing was conducted in 900 ml of dissolution medium hydrochloric acid buffer pH 1.2 (USP 38 NF 33) at 37 ± 0.5 °C using Apparatus II paddle (Varian VK, USA). Apparatus was adjusted to a speed of 60 rpm. Aliquots were taken at regular time intervals (after 5, 10, 15, 20, 30 and 45 min) and replaced with an equal volume of pre-warmed hydrochloric acid buffer. Withdrawn aliquots were analysed for drug content using previously validated HPLC method (Hitachi HPLC system, Japan), column 150 mm x 4.6 mm, 5µm, at 25 °C and flow rate of 1.5 ml/min. Quantification of API was detected by UV at 250 nm.

Results and discussion

LOD as a parameter is extremely important for the process of fluid-bed granulation and drying. Obtained results indicated that sample 2 has the lowest loss of mass (3.1%) in comparison with samples 1 and 3 (3.25 and 3.74%, respectively) probably due to the presence of microcrystalline cellulose into the granule matrix. Even though microcrystalline cellulose allows rapid addition of the granulating fluid, the water does not become bound inside, but rather it is easily given up during drying process. This property aids in preventing case hardening and uniform moisture content in granules (FMC Pharma, 2000).

Results obtained for the flow properties of the final blends indicated that the flowability was excellent for all prepared samples (10, 8.7 and 10.8 sec for sample 1, 2 and 3, respectively).

The macroscopic appearance of the tablets was satisfying. All prepared tablets were smooth and elegant and no mottling was observed. Uniform appearance of the tablets was probably due to presence of microcrystalline cellulose, which enables equal migration of added dyes (FMC Pharma, 2000).

The results of disintegration time show that all prepared samples have disintegration time less than 15 minutes (1.38, 2.35 and 1.21 min for sample 1, 2 and 3, respectively).

Sample 1 did not meet acceptance criteria for immediate release tablet dosage forms (80.22% were dissolved for 30 minutes). On the other hand, sample 2 and 3 were characterized by > 85% dissolved API at the time interval of 30 min (90.06% and 91.82%, respectively). Obtained results pointed to the rationality of microcrystalline cellulose intragranular incorporation (sample 2) as well as addition of croscarmellose sodium (sample 3) equally between the intra and extra granulation.

Conclusion

In the present work efforts have been made to develop immediate release tablets with water insoluble API using fluid-bed granulation as a promising approach to enhance the drug release profile. The results showed that the release of the drug was depended on type of excipient used in the formulation. Formulation containing 2% croscarmellose sodium showed minimum disintegration time and better drug release profile as compare to other formulations.

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Short communication

Evaluation of physical properties on nonsteroidal anti–inflammatory gel formulation with different polymers

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Introduction

Pharmaceutical semisolid preparations may be defined as topical products intended for application on the skinor accessible mucous membranes to provide localized and sometimes systemic effects at the site of application. However, most of the semisolid preparations are applied to the skin for topical relief in dermatologic conditions (Swarbrick et al., 1990). Several categories of semisolid preparations for cutaneous application may bedistinguished: ointments, creams, gels and pastes. These topical formulations are composed of drug in asuitable semisolid base which is either hydrophobic or hydrophilic in character (Allen et al., 2011).

Depending on the physicochemical properties, desired site of action and formulation strategies for the drug delivery incorporation into semisolids can show their activity on the surface layers of tissues or via penetration into deeper layers to reach the site of action or through systemic delivery. Nonetheless if the drug is to act locally or systemically, it must first penetrate the stratum corneum (Raw et al., 2013).

To treat a number of painful conditions affecting the joints and muscles, such as backache, rheumatic and muscular pain, sprains, strains and sports injuries, an active ingredient that belongs to a group of non-steroid anti-inflammatory drug (NSAID) was used. During formulation development, in order to obtain a stable, transparent, homogeneous hydro-alcoholic gel for topical use with satisfactory rheological properties, several gelling agents were tested such as hydroxyethyl cellulose, sodium carboxymethylcellulose, poloxamer and carbomers.

The aim of this research work wasto show the influence of different types of polymers as gelling agents on physical properties of gel, like grittiness, viscosity and spreadability which are important for achieving therapeutic efficiency.

Materials and methods

Materials

NSAID (BCS class II), Poloxamertype 407, Carbomer, grade 940,C10-30 alkyl acrylate crosspolymer,ethanol 96%, diisopropanolamine, sodium hydroxide, propylene glycol, levomenthol.

Equipment

Laboratory mixer homogenizer (IMA Stephan UMC 5,Germany), magnetic stirrer (IKA Ret control/t, Germany), viscometer (Brookfield model DV2T with T- bar spindle, USA), pH meter (Seven Compact Mettler Toledo, Germany),Morphologi-G3S, (Malvern instruments, UK).

Preparation of gels

Method of preparation of samples iskept to be same. Processing steps include hydration of the polymer with mixing until complete hydration is obtained. Next is the gel forming step,where with appropriate gel forming agent clear gelwasachieved. Separately, process of dissolving of active ingredient, levomentholand propylene glycol into ethanol 96% is performed.This solution is added to gel base and homogenized.

Sample S1 contains cross-linked polyacrylate polymer as a gelling agent and diisopropanolamineas a pH balancer. Organic amines are commonly used to neutralize polymers as agents for gel formation. In sample S2, C10-30 alkyl acrylate crosspolymer, polymer of the same group, but with different physical characteristics was used. The sample S3 was prepared with Poloxamer as a gelling agent. So-

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dium hydroxide solution as a pH balancer is used in the optimal amount to obtain a transparent gel.

Propylene glycol and ethanol (96 %) were included in formulation as co-solvent and solvent for the active ingredient. As a medium for hydration and swelling of polymer,water purified was used. Levomenthol in the formulation was chosen as a cooling agent and skin penetration enhancer.

Physical properties of the prepared formulation

The produced gels were evaluated for grittiness, viscosity and spreadability as physical tests.

Grittiness (texture)

The samples were evaluated microscopically for the presence of any appreciable particulate matter, seen under light microscope. Proper quantity from the samples was applied directly to the glass for viewing under a microscope with a thickness that is appropriate for the lens.

The gel should fulfil the requirement of absence of particular matter and from grittiness as desired for any topical preparation.

This test was performed on three selected samples with differentpolymers on Morphologi-G3S Malvern instruments, UK.

Viscosity

Rheological properties such as viscosity of semisolid dosage forms can influence their drug delivery. The viscosity of the formulations was performed using Brookfield viscometer DV2T model with T – Bar spindle, Brookfield. The test was developed according to European Pharmacopoeia test 2.2.10 (Ph. Eur. current version).

For the measurement approximately 50 g of gel were filled in a 100 ml beaker and the T-bar spindlewas lowered perpendicular in the centre taking care that the spindle does not touch bottom of the beaker. The viscosity was read as a single point measurement after 60 s, rotating with 5 rpm at room temperature.

Spreadability test

The spreadability is a test of the gel easiness of application. The spreadability of the samples was determined according to in-house test and is referenced according to Rao et al.(2010) by measuring 1 g gel between horizontal plates (20 x 20 cm^2), after 1 minute. The standardized weight tied on the upper glass was 125 g. The results were calculated according to a formula.

The spreadability (S) can be calculated using formula:

$$S = m \times \frac{l}{t}$$

Where:

S –spreadability(g.cm/sec)
m - weighttied to upper glass slide (g)
l - lengthmove on the glass slide (cm)
t –timetaken (sec)

Results and discussion

For this purpose three different hydro-alcoholic gelswere prepared for testing certainphysical properties.

Grittiness (texture)

All samples have a homogeneous appearance without particulate matter.That is an expected result for this kind of topical formulations.

Viscosity

The results from measurements are 54 000, 41 600 and 700 000 cP for the samples S1, S2 and S3 respectively. Viscosity results were influenced by the gelling properties andmolecular weight of thepolymers.

Spreadability test

Average result from ten measurementsof the samples S1, S2 and S3 were 10.04 g.cm/sec, 10.96 g.cm/secand 5.67 g.cm/sec, respectively. Results are in accordance with the viscosity values, similar between S1 and S2, S3 being least spreadable.

Conclusion

All examined samples for the physical parameter grittiness gave satisfactory results.From the presented results it can be concluded that gel sample with Poloxamer is the most viscous and it has weaker spreadability properties compared to the samples with cross-linked polyacrylatepolymer and C10-30 alkyl acrylate crosspolymer. Samples S1 and S2 have optimal viscosity values, provide good spreadability properties and are easy to apply on the affected skin area.

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Short communication

Taste masking approach in oral suspension with nonsteroidal anti - inflammatory drug

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Introduction

Oral liquid pharmaceutical dosage forms are designed to provide maximum therapeutic response in a targeted population, especially for children and people with difficulty in swallowing tablets and capsules, and to produce rapid therapeutic effects. Suspensions as dispersed systems are composed of two or more phases, where the solid phase, usually the drug substance, is distributed throughout the polymeric matrix (Cox, 2008).

Oral suspension described in this study is composed of active substance which belongs to BCS class II, and is nonsteroidal anti - inflammatory agent with very intensive burning taste, as is literally described as "burning mouth effect". Taste masking effect is very difficult to achieve, but is inevitable step which must be satisfied during development of successful formulation of such oral suspension. Different approaches are reported in the literature to achieve successful taste masking of bitter or unpleasant taste of drug, as follows: addition of flavoring and sweetening agents, taste suppressants and enhancers, viscosity enhancer, pH modifier, microencapsulation, coating with inert material, ion-exchange, inclusion complexation, granulation, adsorption, prodrug approach, by using liposomes, by effervescent agent etc. (Baig et al., 2014; Bhalerao et al., 2013; Suthar et al., 2010).

During formulation development several combination of polymers and ion exchange resins in different ratio are being used. Polymers used for achieving the desired viscosity range were xanthan gum, carboxymethylcellulose sodium, microcrystalline cellulose and carboxymethylcellulose sodium complex, maize starch, lambda carrageenan gum, and cross linked polyacrylic polymer.

Materials and methods

For preparation of the oral suspension, the following materials were used: xanthan gum, carboxymethylcellulose sodium, microcrystalline cellulose/ carboxymethylcellulose sodium , maize starch, lambda carrageenan gum, polacrillin potassium, cross linked polyacrylic polymer, surfactants, flavor, pH regulatory agents, sweeteners and purified water.

All the experimental trials were prepared in several continued steps including polymers hydration, swelling and incorporation of the active substance, using appropriate equipment: table balance (Sartorius CP 4202 S, Mettler PM 200, Germany), stirrer (IKA UltraTurrax T-50 basic, Germany), magnetic stirrer (Heidolph MR Hei-Tec, Germany), viscometer (Brookfield model RV with standard spindle set, Germany), pH meter (Seven Compact Mettler Toledo, electrode Inlab® Solids Pro, Germany).

Seven different formulations were prepared. Combinations of different percentage of xanthan gum, and the same percentage of carboxymethylcellulose sodium and microcrystalline cellulose/ carboxymethylcellulose sodium, were included into 3 experimental trials (F1, F2 and F3). In the fourth formulation (F4), maize starch was incorporated instead of carboxymethylcellulose sodium, and the fifth formula (F5) included lambda carrageenan gum instead of carboxymethylcellulose sodium.

Method of preparation of the above mentioned trials consists of several processes which include preparation of polymer medium with hydration and swelling process under continuously stirring, and after that incorporation of the active substance into polymeric matrix. Additional inactive ingredients which enhance the organoleptic properties and consistency of the suspension were added, such as sweetening agents, flavors, pH modifiers and surfactants.

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Into the last two formulations (F6 and F7) ion-exchange resins were included. They were prepared with complexation process between the ion-exchange resins and the active substance.

Viscosity measurements were done of all of the prepared experimental trials using laboratory viscometer (Brookfield DV2T RV with standard spindle set, Germany).

The experimental conditions (600 ml beaker, filled with 350 ml suspension, viscosity measured after 60 seconds at 50 rpm of spindle rotation) are kept the same for all of the performed trials which means that the obtained results are comparable with each other.

The palatability study for the prepared experimental trials was performed by panel method. The study protocol was explained and written. Also, the consent was obtained from the volunteers. For this purpose, 10 human volunteers were selected. About 5 ml of the suspension was placed on tongue and taste evaluated after 15 seconds, using a numerical scale. The numerical scale consists of values as 0 = excellent, 1 = good, 2 = slightly burning mouth effect, 3 = burning mouth effect, 4 = intensive burning mouth effect, which were determined by the formulator.

Also, measurement of the sedimentation volume of all of the experimental trials in the study was done. Sedimentation volume (F) is a ratio of the final or ultimate volume of sediment (Vu) to the original volume of sediment (Vo) before settling. It can be calculated by following equation:

$$F = V u / Vo$$

where,

Vu = final or ultimate volume of sediment

Vo = original volume of suspension before settling

Results and discussion

In the formulations with ion – exchange resins, interaction between the reactive functional group of the polyacrylic polymer and ionisable active substance molecule occurs, until active substance - polymer complex is formed. The reaction is performed under determined pH and temperature values. The complex is insoluble in water and it has no taste, so the bitter taste of the active substance entrapped into the complex is masked.

When such complex enters the gastro intestinal fluid, the bond between the active substance and polymer diffuses and the molecule of the active substance is released throughout a decomplexation process. The process of releasing the active substance is performed very quickly and completely into gastro intestinal fluid so it does not affect the absorption and bioavailability of the active substance. (Bilandi et al., 2014; Sampath Kumar et al., 2012).

Different values for the viscosity measurement of all of the experimental trials were obtained, which logically were in correlation with the used percentage of the selected polymer. Values from viscosity measurement for the formulations F1, F2 and F3 were 1046 cP, 1234 cP and 1748 cP, as follows. For the trials F4, F5, F6 and F7 were obtained the following values 1040 cP, 1730 cP, 738 cP and 1916 cP, respectively.

The sedimentation volume can have values ranging from less than 1 to equal or rarely, more than 1. The ultimate height of the solid phase after settling depends on the concentration of solid and the particle size. To obtain an acceptable suspension the value of F should be at least 0.9. In the presented study there is no sedimentation after 14 days for all of the seven trials.

Conclusion

From the performed experimental trials it can be concluded that increasing the viscosity of the prepared oral suspension with combination of different percentage of xanthan gum, and the same percentage of carboxymethylcellulose sodium and microcrystalline cellulose/carboxymethylcellulose sodium, results into satisfactory taste masking effect which is more intensively achieved in comparison with the rest of the experimental trials. The complexation process does not give a satisfactory taste masking effect, although these formulations were optimistic and promising.

The combination of the three polymeric matrices in the optimized concentration, which results into satisfactory viscosity of the suspension containing nonsteroidal anti - inflammatory active substance, was chosen as a favorite formulation and a candidate to work with in the future trials.

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Influence of formulation variables on encapsulation efficiency of microsponges

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Introduction

Pharmaceutical/cosmetic industry trends are focused toward development of innovative formulations characterized by controlled release of active ingredient (AI) in order to enhance effectiveness and to reduce AI related side/adverse effects.

Microsponges (MSPs) incorporated into well known dosage forms represent new generation of highly effective formulations. MSPs efficacy is related to particle size and size distribution, porosity, pore size, surface area, AI encapsulation efficiency (EE) and release rate. MSPs` characteristics (physico-chemical, biopharmaceutical) influencing their efficacy might be tailored using certain preparation technique, as well as formulation and process parameters. Effect of formulation variables upon MSPs` EE is going to be presented in this review. Quasi-emulsion solvent diffusion technique was most commonly used method for MSPs preparation, while drug to polymer ratio and composition of internal and outer (external) phase were identified as significant formulation variables that influenced EE.

Influence of drug to polymer ratio

EE of Ethyl cellulose (EC) or Eudragit RS100 (ERS100) based MSPs loaded with fluconazole (Abdelmalak and El-Menshave, 2012), was in a range of ~15 to 90% depending from the variables studied. Results from their study pointed that increased polymer quantity resulted with higher EE (~45% *vs* 55% for 1:1 and 1:2 drug to polymer ratio (DPr), respectively). According to authors the findings might be related to the fact that higher polymer amount would result with increased viscosity of internal organic phase (IOP), thus reducing drug molecule diffusion into the external phase (EP) or simply higher amount of polymer encapsulated more drug molecules.

Contrary, studies related to ERS100 MSPs loaded with diclofenac diethylamine (DPr 1:1 to 1:6) (Osmani et al., 2015a) or domperidone (DPr 1:1 to 1:5) (Osmani et al., 2015b) showed opposite trend. Determined diclofenac diethylamine EE values ranged ~10% for DPr 1:6 to 48% for DPr 1:1 (Osmani et al., 2015a), while domperidone EE were ~73% (DPr 1:5) to 92% (DPr 1:1). Similar were the findings for benzoil peroxide EC MSPs (Jelvehgari et al., 2006) prepared with DPr of 1:1 to 13:1 characterized by EE of 70-98%, whereas probably higher drug quantity would result with increased EE as more drug molecules per polymer unit are available.

Studies of Çomoğlu at al. (2003), Orlu et al. (2006) and (D'souza and More, 2008) related to EE of ERS100 MSPs loaded with ketoprofen (DPr 1:1 to 11:1, EE ~92-96%), flurbiprofen (DPr 3:1 to 5:1, EE ~96-97%) and fluocinolone acetonide (DPr 1:1 to 13:1, EE ~87-94%) accordingly, indicated that EE was not much affected by DPr.

Studies of Arya and Pathak (2014) and Srivastava et al. (2012) associated to ERS100/EC and ERS100 MSPs loaded with curcumin and meloxicam, respectively, were conducted using experimental design studies. EE were ~80-93% for curcumin ERS100/EC MSPs and ~71-99% for meloxicam ERS100 MSPs. Derived correlations between EE and studied variables - EC amount (300-900mg) in the IOP and Polyvinil alcochol (PVA) (0.5-1.5% w/v) in the EP (Arya and Pathak, 2014) and volume of organic media - dichloromethane (DCM)(5-7 ml) and ERS100 content (400-

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1200 mg) in DCM (Srivastava et al., 2012) pointed to the positive influence of polymer quantity, although later notified that wasn`t statisticaly significant as it was expected. However, influence of interactions` factors upon EE was also identified. Similar findings were observed in the study of Gupta et al. (2015) where 5-fluorouracil ERS100 MSPs were prepared using different polymer quantity (400-1200 mg) and IOP volumes (6-8 ml) consisted of ethanol:DCM mixture (7:3) (EE ~36-61%) as well as in the study of Pawar at al. (2015) related to oxybenzone EC MSPs prepared with DPr 1:1 to 1:3 and DCM - 3-5 ml (EE ~90-99%).

Influence of organic phase

Influence of solvent type (ethanol, methylen chloride) upon MSPs` EE was investigated by Abdelmalak and El-Menshave (2012) where determined EE indicated that ethanol was solvent of choice most likely due to the higher boiling point and hence lower evaporation rate thus decreasing the diffusion rate into the EP. Orlu et al. (2006) used 3, 5 and 10 ml ethanol for MSPs preparation, but however MSPs could be prepared only with 3 ml ethanol as IOP. When volume of DCM was increased from 5 to 15 ml in the study of Jelvehgari et al. (2006) a decrease of EE was observed (~87 to 67%), most likely due to the lowering of drug concentration.

Higher EE values in increase of IOP volume were determined by Gupta et al. (2015) and Pawar et al. (2015) probably due to the better drug solubility, but however inverse correlation for interaction term of polymer amount and IOP volume was observed. Alike were the results of Srivastava et al. (2012) where additionally quadratic functions of investigated variables` influence upon EE were determined. Inverse dependency of EE to $(IOP)^2$ might be linked to the formation of more porous MSPs thus facilitating drug partitioning into the EP.

Influence of surfactant concentration in the outer water phase

Curcumin EE of ERS100/EC MSPs (Arya and Pathak, 2014) showed positive correlation with the interaction of EC and PVA, while negative correlation was established with the influence of quadratic terms (EC^2*PVA, EC^2*PVA^2). Influence of PVA on the EE was also determined in the study of Jelvehgari et al. (2006) where obtained results pointed that benzoil peroxide EE was higher at lower PVA concentration (EE of 93.26% at 0.047% PVA and 77.87% at 0.187% PVA). Similar results were reported by Abdelmalak and El-Menshave (2012) where fluconazole EE was inversely dependent from PVA concentration (EE ~40% at 0.75% PVA to > 70% at 0.5% PVA). These observations were explained by probable formation of alternative hydrophobic regions dissolving some drug portions thus resulting with decreased EE.

Opposite findings were determined when PVA solution in a concentration of 0.03 to 0.07% was used as EP for preparation of domperidone ERS100 MPSs (Osmani et al., 2015b) and hence EE was ~76 to 90%, respectively. The difference with the previous studies might be related to the low PVA concentration used. Similar were the findings for diclofenac diethylamine ERS100 MPSs (Osmani et al., 2015a) prepared with sodium alginate solution (0.03 to 0.07%) as EP which were characterized by slight increase of EE (~85 to 93%), consequently.

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Short communication

Optimization of viscosity building agent in oral paediatric suspension

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Introduction

Suspensions are two-phase systems having solid, substantially water insoluble active agent particles dispersed throughout liquid medium. They represent thermodynamically unstable disperse system, so it is necessary to add suspending agent which reduces the rate of settling and permits easy redispersion of any settled particulate matter, both by protective colloidal action and by increasing the consistency of the suspending medium. Gums and other polymers are frequently employed in pharmaceutical suspensions as viscosity building, thickening, stabilizing and suspending agents. They are used to delay or prevent sedimentation by affecting the rheological characteristics of suspensions. Viscosity building agents increase the viscosity of an aqueous mixture without substantially modifying its other properties, such as taste. They provide body and improve stability of added ingredients (Moreton, 2010).

The aim of the study is optimization of viscosity building agent, xanthan gum in combination with hydroxyethyl cellulose in order to achieve stable oral suspension. In order to evaluate the optimal concentration of the viscosity building agent, formulations with different concentration of xanthan gum were made. Appearance and homogeneity of suspension, viscosity, sedimentation volume and dissolution were evaluated.

Materials and methods

Materials

Active pharmaceutical ingredient, sparingly soluble in water, para-aminophenol derivative that exhibits analgesic and antipyretic activity with bitter taste was used. Solvents, co-solvent, stabilising agent, sweetening agent, buffering agent and preservative were used. Xanthan gum (Jungbunzlauer, Austria) and hydroxyethyl cellulose (Ashland, France) were used as viscosity building agents. Flavour and taste masking agent were used to mask the bitter taste of the active ingredient. All excipients are approved for use in pharmaceutical industry.

Methods

Suspensions were produced by a common process for preparation of oral suspension by method of mixing without heating, dispersing of active ingredient and homogenization. Each of the laboratory trials was prepared under the same condition and with the same method. Suspensions were prepared with different concentration of xanthan gum 0.15%; 0.17%; 0.20%; 0.25% and 0.35%. The concentration of hydroxyethyl cellulose was kept constant in all five laboratory trials. Visual inspection for appearance and homogeneity of suspension, viscosity, sedimentation volume and dissolution was evaluated as critical parameters.

Homogeneity of the prepared suspensions was evaluated using optical microscopy (Morphologi-G3S, Malvern instruments, UK). Viscosity of the suspensions was measured with rotating viscometer (Brookfield, USA, with RV spindle No. 3; temperature 20ºC). Sedimentation volume is defined as the ratio of the final, equilibrium volume of the sediment to the total volume before settling (F = Vu /V0 x100; Vu – volume of sediment in ml; V0 – total volume of suspension in ml). The values for F range from 0 to 1. Values of F = 1 indicate that no sediment is apparent and that the suspension is stable and flocculated (Mohammad et al., 2010). Sedimentation volume was observed and calculated for a period of 30 days, in the following intervals: 60 minutes; 180 minutes; 24 hours; 7 days and 30 days. Content of dissolved active component was determinate with

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HPLC method. Dissolution testing was conducted in 900 ml + 1% phosphate buffer pH 6.8+ 0.05 using USP apparatus II (paddle), set at rotation speed of 50 rpm and temperature of the medium of 37 ± 0.5ºC.

Results and discussion

Homogeneity of the prepared suspensions was evaluated microscopically and differences due to different concentration of viscosity building agent were not observed. Suspensions with concentration of xanthan gum in range from 0.15%; 0.17%; 0.20; 0.25% to 0.35% were measured and results from 562 cP; 687cP; 752 cP; 1005 cP to 1233 cP, respectively for each concentration, were obtained. The increase of the concentration of xanthan gum resulted with an increase of viscosity of the suspensions, which was easily visually noticed and after that confirmed by measurement with a rotating viscometer. The suspension with viscosity above 1000 cP showed bad pourability which can directly influences the proper dosing of the oral suspension (Zatzx and Knapp, 1984). The suspensions were observed during a period of 30 days and sedimentation effect was not observed. Sedimentation volumes for all evaluated concentrations were 1, indicating that xanthan gum used in a range from 0.15% to 0.35% is capable of forming and maintaining a stable suspension for this period. The increase in viscosity avoids the particle aggregation and helps particles to remain in a flocculated state (Tempio and Zatz, 1980). Dissolution testing was performed on the prepared suspension and the following results were obtained 98.22%, 95.15%, 92.22%, 89.42% and 67.95% for each concentration respectively, for the time point of 30 minutes and 99.52%, 98.21%, 97.36%, 91.50% and 81.65%, for the time point of 45 minutes. The percent of dissolved active component decrease by increasing the concentration of xanthan gum, or viscosity of the suspensions. Obtained results from dissolution of the suspensions were higher for the time point of 45 minutes in comparison with the results for 30 minutes. From the results can be noticed that due to its polysaccharide nature the viscosity building agent has the ability to decrease the dissolution rate of the active component when added in higher concentration (Verhoeven et al., 2006). As the viscosity of the dispersion medium increases, the terminal settling velocity decreases thus the dispersed phase settle at a slower rate and remain dispersed for longer time yielding higher stability of the suspension. On the other hand as the viscosity of the suspension increases, it's pourability decreases and the inconvenience to the patients for dosing increases. Also the increase of the viscosity resulted in decrease of the dissolution rate of the active component, directly influencing the bioavailability of the drug.

Conclusion

With concentration optimization of viscosity building agent xanthan gum, viscosity of the suspension can be maintained within optimum range to yield a homogenous, stable suspension appropriate for oral delivery of the drug.

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Short communication

Risk assessment of excipients in medicinal drug products: a short review

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Introduction

Pharmaceutical excipients are substances other than the active pharmaceutical ingredient(API), which are intentionally included in a drug delivery system. They are inert substances by definition but in many cases represent high proportion of the finished dosage form and their quality significantly influences the effectiveness or safety of the drug. Excipients support the processing of the finished dosage form during its manufacture; they protect, support or enhance stability, bioavailability or patient acceptability; help in product identification, or enhance other attribute of the general product quality attributes.

Despite their critical role in the pharmaceutical formulation, there are no specific GMP regulations obligatory for their manufacturers. On the other hand, there are a huge number of applications of these materials which makes the development of appropriate guidelines very demanding. Currently, in the pharmaceutical industry there is a general expectation that excipients are manufactured to recognised GMP principles (The Joint IPEC – PQG Good Manufacturing Practices Guide for Pharmaceutical Excipients, 2006).

The major problem for Marketing Authorization Holders(MAHs) is that the excipient manufacturers produce ingredients that are not only intended for use in pharmaceuticals but also in food, cosmetics, or as chemicals. The quantitative requirement for excipients in pharmaceutical products is often insignificant compared to their use in other applications; however, the quality of the ingredients for use in medicinal products could be essential to the safety, quality and efficacy of the finished drug product (IPEC Federation Position Paper on EU Risk Assessment Guidelines for Excipients 2015C/ 95/02).

Most important global organizations which instruct and facilitate implementation of excipient quality standards are EDQM (European Directorate for Quality of Medicines and Healthcare) and the IPEC (International Pharmaceutical Excipients Council).

EDQM position

At the moment it is the Eudralex Vol. 4, Part 2 guideline, which refers to the quality system of API manufacturers and is sufficient and even higher standard for the excipient manufacturers.

According to this guideline excipients and excipient manufacturers should be controlled based on the results of a formalised quality risk assessment (EudraLex, Volume 4 Good manufacturing practice (GMP) Guidelines, Part 1, Chapter 5: Production). The MAHs are required to have a documented risk assessment/management system for appropriate GMPs for excipients, available on site for review by EU GMP inspectors.

According to The Guideline of 19 March 2015 on the formalised risk assessment for ascertaining the appropriate good manufacturing practice for excipients of medicinal products for human use, the risk assessment principle consists of three parts:

1. Determine the appropriate GMP level that the excipient manufacturer needs to achieve;
2. Determine the current GMP level that the excipient manufacturer has;
3. GAP analysis between these two GMP levels and proposing actions.

The following specific areas of potential risks should be considered, understood and assessed when reviewing the excipient manufacturers' GMP level: risk aspects related to excipient quality and safety, and risk aspects related to excipient function in the drug product.

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Once the appropriate GMP for the excipient and the risk profile of the excipient manufacturer have been defined, the EU guidelines specify that ongoing risk review should be performed.

IPEC's position

The newest and customized guideline for excipient manufacturers that covers topics from the EU GMP guidelines and ISO 9001 chapters is The Joint IPEC – PQG Good Manufacturing Practices Guide for Pharmaceutical Excipients, 2006, which represents good base for implementation of the highest GMP for the excipient manufacturers.

The major concern is that the risk assessment process by different MAHs could eventually classify one manufacturer into different categories of risk (high, medium, low) depending on the different approaches and intended use. This could lead to different quality requirements by different companies for the same excipient. It would be impossible to comply with these different requirements in one quality system unless the highest level of GMP was to be implemented. IPEC is concerned that there is not enough time to complete risk assessments for all excipients by the March 21, 2016 deadline, and that incomplete assessments may jeopardize the availability of high–quality excipients that have been in use for many years.

IPEC will share their views with the EDQM on the challenges to comply with the current timeline and request more realistic goals and timelines (IPEC Federation Position Paper on EU Risk Assessment Guidelines for Excipients 2015/C 95/02).

IPEC views third−party auditing and certification schemes, such as EXCiPACT, and national standards, such as NSF/IPEC/ANSI 363−2014, as playing an essential role to achieve compliance with new requirements for the qualification of excipients and their suppliers. Without additional information about GMP and GDP compliance of the excipient manufacturers through independent third−party audits, it will be nearly impossible for MAHs on their own to gather all the necessary data required (Quality Risk Assessment for Excipients: An Industry Perspective, 2015).

Regulatory authority's position

Even though the groundwork for standardization is established, there is a concern from the regulatory authorities: Ewan Norton of the UK Medicines and Healthcare products Regulatory Agency (MHRA) gave an inspector's view of the new EU requirements on risk−assessment of excipients, expressing concern that despite a 12−month introduction period some pharma companies may not be ready to meet the deadline of 21 March, 2016.

A survey carried out at an MHRA conference last year revealed that a considerable number of pharma industry had either not started the risk assessment process or were unaware of the upcoming requirements. Norton suggested that inspectors would be inclined to serve companies with a deficiency in their report if they were not compliant with the requirements (Excipients insight January/February 2016, IPEC e−newsletter, 2016).

Pharmaceutical industry position

EFPIA (European Federation of Pharmaceutical Industries and Associations) supports the new legislation but is concerned that its implementation may lead to an abundance of regulatory guidance. EFPIA recommends that instead of formalized risk assessment the process should be integrated into existing supplier qualification management programmes. They recommend that it is not necessary to develop additional GMP guidelines for excipients since the risk management principle is already embedded in Part III EU GMP: Quality Risk Management (ICH Q9).

They propose more effective legislation without unnecessary regulatory burden on manufacturers and suppliers to ensure continuous supply of quality excipients and finished drug products (EFPIA TDOC Position Paper).

Conclusion

In the literature, depending on the standpoint there are an abundant number of guidelines, papers and articles that explain the general recommendations for risk assessment principle, but lack those describing hands−on application. There are a limited number of case studies of the pharmaceutical companies applying those guidelines and showing significance of those guidelines and practices.

It is recommended that the literature invests more in the area of application and significance of guidelines and practices. New case studies should be done to prove the success of such practices in risk assessment of excipients.

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Hold-time stability study - a "must-do" for pharmaceutical industry

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Introduction

Three crucial characteristics of medicinal product are its safety, effectiveness and quality required for its intended use. In order to obtain and retain those characteristics, special consideration must be paid to proper storage of medicinal product. Good Manufacturing Practice (GMP) requires that raw materials, packaging materials, intermediates, bulk and finished product are stored under appropriate conditions. This also means that a maximum holding period for intermediates and bulk products before their further processing should be established. In order to avoid any misunderstanding, it is important to define terms "intermediates" and "bulk products" The term "Intermediate" is partly processed product that must undergo further manufacturing steps before it becomes a bulk product. "Bulk product" refers to any pharmaceutical product that has completed all processing stages up to, but not including, final packaging (Eudralex, 2010).

Hold-time studies guidance

Until 2015, relevant guidelines (CPMP/QWP/122/02, 2003; ICH Q1A(R2), 2003; Eudralex, 2014; FDA Q1A(R2), 2003) thoroughly described the requirements for stability testing of finished drug product. The only statement in guidelines with regard to pharmaceutical bulk products or intermediates is that they have to be stored "in a suitable way".

In 2015, World Health Organization (WHO) released a final version of "General guidance on hold-time studies" as a part of WHO Technical Report Series No. 992 (WHO, 2015). Although this guidance describes the principles of establishing criteria for performing hold-time studies on coated tablets, those principles can be applied to other non-sterile dosage forms.

Recommendations for conducting hold-time studies

Hold time is a period of time during which materials can be stored under defined conditions and will retain their quality within the specified limits. This implies that hold times should be established for the materials at different phases of manufacturing, so one can be sure that holding didn't compromise the quality of the finished product.

Hold times should be established based on scientific data. It is recommended that the test is to be performed on one batch prior to registration of the product. All tests should be performed using validated stability-indicating methods. Storage conditions of samples are required to be the same as for the quarantine area or manufacture stage (WHO, 2015). Apart from that, sample should be stored in a simulated package mimicking the packaging of the bulk product (Huynh-Ba, 2009; WHO, 2015). This means that containers should be made of the same material and using same closure system as the system in the manufacturing stage. If there is a risk of substance's degradation as a result of oxidation processes, headspace in the containers should also be tested. It is necessary to determine the ratio of headspace to contents in the containers, so degradation of substance is unlikely to occur (WHO, 2015).

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Data to support holding times could be collected during drug development, on a pilot or validation batches. If the product is already on the market, guidance (WHO, 2015) leaves a possibility to perform retrospective hold-time studies. Collected data should be processed statistically in order to note trends or to establish limits. Study should be performed through a period defined in the guidance, and not beyond that. It isn't necessary to prolong study until the lowest limit of quality is achieved. This approach is based on a "most probable" as opposed to "worst case" approach. For example, 90 days are enough time to keep core tablets as intermediates in the coated tablet manufacturing, to study hold-time stability (WHO, 2015).

General guidance on hold-time studies (WHO, 2015) provides examples of stages, tests and study times that may be performed for a coated tablet. Coated tablet have been chosen as an example, since tablets are the most widely used dosage form. Apart from that, manufacture of tablets and, especially, coated tablets can be a complex process, including a variety of manufacturing stages (Armstrong, 2007). Each stage can, in some way, influence the quality of the final product.

Once hold time is established, intermediates and bulk products should not be stored beyond this period.

Conclusion

Hold-time study plays an important role in the manufacturing of drug products in the GMP environment. The maximum hold time for intermediates and bulk pharmaceuticals should be established in order to continue their processing or start the packaging of the drug products before their quality becomes compromised.

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Comparison between some methods for solubility enhancement of lorazepam

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Introduction

Lorazepam has anxiolytic, sedative, hypnotic, and anticonvulsant as well as muscle relaxant properties. It is a highly lipophilic compound whose partition coefficient is 2.36 when measured at 20 °C. Lorazepam, as an amphiphilic substance, has two pKa values (1.3 and 11.5) determined spectrophotometrically in aqueous buffers. It is mainly used in different dosage forms as an active substance. The injectable solution comes in 1 mL ampoules containing 2 or 4 mg lorazepam. As derivative of 1,4-benzodiazepines, has certain limitations in pharmaceutical technology due to its poor solubility in water. The aqueous solubility of lorazepam, particle size 8.9 μm, is 0.0485 mg/mL (Hadžiabdić and Hadžović, 2005-2006). Often, in a certain volume of water, an adequate concentration of the drug cannot be achieved during formulation of a liquid dosage form due to the low solubility of the drug (Jinal et al., 2012).

This study investigated the use of the traditional solubilization approaches to increase the solubility of lorazepam. Our aim was to investigate the solubility of lorazepam in phosphate buffer solutions, in water/cosolvents mixtures (cosolvents: ethanol, propylene glycol, polyethylene glycol 200 and 400) (Corrigan and Healy, 2007; Vemula et al., 2010), and in water/surfactants mixtures [surfactants: Tween 80, Tween 20, Brij 35, sodium cholate, sodium deoxycholate, sodium taurocholate] (Corrigan and Healy, 2007; Rangel-Yagui et al., 2005) as well as its solubility in water/cyclodextrins mixtures [cyclodextrins: α-cyclodextrin (α-CD), β-cyclodextrin (β-CD), 2-hydroxypropyl-β-cyclodextrin (2-HP-βCD)] (Loftsson and Brewster, 2010; Sathesh Babu et al., 2008). The main objective was to find the most suitable method for providing good solubility of lorazepam and thus its formulation in a liquid dosage form.

Materials and methods

Materials

Used chemicals were obtained from: Lorazepam (LZ, Sigma-Aldrich, Germany); Acidum sulfuricum 95-97% and Methanolum (Kemika, Croatia); Acidum hydrochloricum 37%, Disodium hydrogen phosphate and Potassium dihydrogen phosphate (Alkaloid, Macedonia); Acidum phosphoricum 85% (Fluka, Chemika, Switzerland); ethanol 96%, Polyethylene glycol 200, Polyethylene glycol 400 and Propylene glycol (Sigma-Aldrich, Germany); Tween 20, Tween 80 and Brij 35 (Merck, Germany); Sodium cholate, Sodium deoxycholate, Sodium taurocholate, α-CD, β-CD and 2-HP-βCD (Fluka, Chemika, Switzerland).

Solubility studies

The solubility of LZ was estimated by the solubility method of Higuchi and Connors (1965). Solubility measurements and the determination of saturation concentrations were carried out by adding excess amounts of lorazepam to phosphate buffer solutions (pH 2.0-8.0), water/cosolvent mixtures (concentrations 1-60% w/w), water/surfactants mixtures (concentrations 1-20% w/w, for anionic surfactants and 1-35% w/w, for non-ionic surfactants). Concentrations of cyclodextrins were selected based on

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their solubility in water. Testing of LZ solubility with the addition of α-CD in aqueous solutions was carried out by its addition in concentrations up to 14%. The testing of LZ solubility was carried out in concentrations up to 1.8% of aqueous solutions of β-CD. The testing of LZ solubility with the addition of 2-HP-β-CD in aqueous solutions was carried out by its addition in concentrations up to 40%. The concentrations of dissolved LZ in tested solutions were determined using a Shimadzu UV-1601, UV VIS spectrophotometer (Shimadzu, Japan).

Results and discussion

It was found that LZ solubility was decreased with an increasing pH. When the pH value is lower, the percentage of protonation of lorazepam is higher. Protonation at low pH occurs at the nitrogen atom in position 4. Deprotonation occurs at a high pH with the loss of the hydrogen atom from the 3-hydroxyl group (Barrett et al., 1973).

To prepare the aqueous solution of LZ (2 mg/mL), it is necessary to have more than 50% (w/w) of propylene glycol, or more than 40% (w/w) of polyethylene glycol 400, polyethylene glycol 200 or ethanol. To completely dissolve 4 mg/mL LZ in water more than 50% (w/w) of polyethylene glycol 400 or polyethylene glycol 200 or less than 50% (w/w) of ethanol is needed. It is evident that LZ molecules preferably solubilize in a nonpolar environment rather than polar (aqueous) surroundings. The concentration of the solvents that enable this drug to be solubilized in water is far higher than that allowed for daily intake.

Increase of surfactants concentration in water leads to LZ solubility enhancement linearly. The best solubility was achieved with sodium taurocholate, of all the tested bile salts, while the best LZ solubility in water was achieved with Brij 35, of all the tested non-ionic surfactants (Alkhamis et al., 2003; Rangel-Yagui et al., 2005).

To dissolve 2 mg/mL of LZ in water, more than 15% of sodium cholate, more than 5% of sodium deoxycholate, or less than 10% of sodium taurocholate concentrations are needed. To make a LZ concentration in liquid form of 2 mg LZ/mL of water, more than 5% of Tween 80, more than 7% of Tween 20, or less than 3% Brij 35 is needed. To dissolve 4 mg/mL of LZ in water, more than 20% of sodium cholate, ~15% of sodium deoxycholate or less than 15% of sodium taurocholate is needed. To dissolve 4 mg of LZ/mL of water, 20% of Tween 80, more than 15% of Tween 20 or less than 7% Brij 35 should be used. The surfactants with higher HLB values were better solubilizers.

The increase of LZ solubility in aqueous solutions of chosen cyclodextrins, compared to its solubility in water. The solubility of LZ is increased 6.5-fold at 14% of α-CD, 4.38-fold at 1.8% β-CD, and 170.1-fold at 40% of 2-HP-β-CD. The changes in the solubility of LZ resulting from the addition of various concentrations of α-CD, β-CD and 2-HP-β-CD were used to plot phase solubility diagrams and to evaluate the stoichiometry and stability constant of the resultant complex. The phase solubility diagrams obtained are linear and show characteristics of AL-type of solubility curve. This is attributed to the formation of a soluble complex. The apparent stability constants (K1:1) were estimated from the straight line of the phase solubility diagrams according to equation of Higuchi and Connors (Higuchi and Connors, 1965; Mosher and Thompson, 2007). In this case, the K1:1 values obtained followed the order 2-HP-β-CD > β-CD > α-CD, reflecting the greater affinity of modified cyclodextrin for LZ compared with their parent α-CD and β-CD.

Conclusion

Based on the results, the changes in the pH value of the media do not lead to a greater solubility of lorazepam. Of the cosolvents used, the greatest increase in solubility of lorazepam in water was achieved with ethanol. Of the bile salts used, sodium taurocholate showed the best solubilization ability, while Brij 35 was the best of the non-ionic surfactants. The solubility of lorazepam with the cyclodextrin derivative, 2-hydroxypropyl-β-cyclodextrin was better than natural cyclodextrins. Surfactants have the highest ability of solubilization of lorazepam in water. Thus, the study generates an array of data for solubilization of lorazepam using various pharmaceutically accepted techniques which could be useful while formulating liquid dosage forms of lorazepam.

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Short communication

Comparison of biopharmaceutical properties of 5-FU loaded TEOS and TEOS/APTES microparticles for colon targeting

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Introduction

Colon drug targeting is valuable approach for the treatment of serious GIT diseases like ulcerative colitis, Chron's disease, colon carcinomas and infections; considering that high local concentration at the site of action can be achieved, and systemic exposure to the active substance as well as serious side effects can be avoided (Chourasia and Jain, 2003). Different studies point to the connection of the inflammation and tumorigenesis and the presence of immunoinflammatory mediators and inflammation in virtually all steps of colon cancer development, including initiation, promotion, progression, and metastasis. Therefore, the design concept of increased localization of micro and/or nanoparticles at the site of inflammation/cancer due to enhanced permeability and retention in the tissue damaged by inflammation/cancer may also be favourable for colon cancer targeting. Additionally, prolonged intimate contact with the epithelial membranes at the site of action, which may be enhanced by incorporation of bioadhesive polymers into the carriers is crucial for improvement of specific interaction with mucin and cell surfaces as well as for improved localization of the drug delivery systems at the site of action. Whence, the aim of this study was to design 5-fluorouracil loaded organomodified silica microparticles as systems for controlled and site specific colon delivery as well as to investigate the influence of the concentration of the silane coupling agent 3-aminopropyltriethoxysilane (APTES) upon the microparticle properties.

Materials and methods

Materials

Silica microparticles were prepared using the following reagents: tetraethoxysilane (TEOS; Sigma, Germany), 3-aminopropyltriethoxysilane (APTES; Sigma, Germany), ethanol 96% (v/v) (Merck, Germany), acetic acid (Merck, Germany), 5-Fluorouracil (5-FU; EBEWE Pharma, Austria), and deionised water. All other used chemicals and reagents were of analytical grade.

Preparation of silica microparticles

Silica microparticles were prepared from tetraethoxysilane (samples MP1) and tetraethoxysilane co-hydrolyzed with 3-aminopropyltriethoxysilane by combining sol-gel technology with spray-drying. TEOS-based silica microparticles (MP1) were synthesized by sol-gel method at room temperature using a one-step acid-catalized hydrolysis. Silica sol was prepared by hydrolysis and polycondensation of TEOS with deionised water, ethanol and acetic acid as a catalyst. The molar ratio of the silica sol was TEOS: water : ethanol : acetic acid = 0.01 : 1.39 : 0.43 : 0.009. Hydrolised silica sol was spray dried with a mini spray dryer (B-290, Büchi Labortechik AG, Switzerland). 5-FU was added after completed hydrolysis and incorporated in microparticles in network synthesis phase at concentration 12 wt%. For partial substitution of TEOS, 2.5 mol% (MP2) and 5 mol% (MP3) organomodified alkoxide was used. 5-FU was dissolved in the hydrolised sol before spray drying at concentration of 12 wt%.

Characterization of silica microparticles

Prepared silica microparticles were characterized in a terms of mean particle size and particle size distribution

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(Mastersizer 2000, Malvern Instruments Ltd., UK), surface morphology (SEM; Jeol-SEM6400, Japan) and drug loading efficiency (HPLC Agilent 1200 with UV detector).

Ex vivo mucoadhesion studies was performed and the percent of mucoadhesion was determined with modified everted sac method (Santos et al., 1999).

In vitro drug release studies

The dissolution test from prepared silica microparticles were carried out in closed glass tubes at 37 °C and 50 horizontal strikes/min (horizontal shaker; Shaker Unitronik OR, Selecta. Spain). To compare the drug release under different pH conditions, 50 mg of the microparticles mixed with 10 ml buffer solution with pH 1.2 and 10 ml buffer solution with pH 7.4. At appropriate intervals, 2 ml of samples were withdrawn, filtered through 0.45 μm membrane filter, and assayed by HPLC method.

In order to investigate the possible drug release mechanisms, data obtained from *in vitro* drug release studies were analyzed using different kinetic models (Higuchi equation, the traditional power law and modified power law) (Viitala et al., 2007). Determined *n* value indicated most probable drug release mechanisam, while goodness of fit was evaluated using the *r* (correlation coefficient) values.

Results and discussion

Prepared particles were spherical with smooth surfaces and unimodal narrow size distribution. Median volume diameter of microparticles was 1.3876 μm (SPAN factor 1.745), 2.1405 μm (SPAN factor 1.133) and 3.3763 μm (SPAN factor 1.713) for MP1, MP2 and MP3, respectively. The content of 5-fluorouracil was 89.5 mg (MP1), 145.71 mg (MP2) and 160.78 mg 5-FU/g microparticles (MP3). The experimental results suggest that addition of precursor 3-aminopropyltriethoxysilane in the silica sol influenced the inner structure of the matrix, resulting with increased porosity and hydrophilicity of the network, resulting in increased average paricle size and drug loading efficiency.

The results from the *in vitro* drug release studies of TEOS based microparticles showed that the silica matrices are capable for controlled release of 5-FU during prolonged time periods. However, the dissolution rate was proportional to 5-FU loading, most probably because of possible strong 5-FU – silica interactions that could initiate formation of matrix microdeformations during the sol-gel process, resulting in overall increase of matrix porosity (Djurdjic et al., 2011). pH dependent drug release was noticed from TEOS based particles with lower percent of 5-FU (2 and 6wt%) (Djurdjic et al., 2010, 2011), but pH dependency was lost when higher percent of 5-FU (12 wt%) was incorporated in the processed silica sol during production of the microparticles, most probably because of increased matrix porosity. The drug release data showed the best fit to modified power law equation; r^2 were 0.967 and 0.963 at pH 1.2 and pH 7.4, respectively for sample MP1. Values *n* for sample MP1 (0.07 and 0.09 at pH 1.2 and pH 7.4, respectively) point to drug diffusion through very porous matrix without capacity for control of the release during time.

Organically modified microparticles showed increased burst and increased drug release rate compared to TEOS based microparticles. Burst release varied accordingly to the percent of APTES. For formulations with highest percent of APTES and drug loading, the total ammount of incorporated 5-FU was released within few hours. The drug release data of organically modified microparticles show the best fit to modified power law model and r^2 were 0.859 and 0.955 (at pH 1.2 and pH 7.4, respectively) for MP2. Values *n* for sample MP2 (0.16 and 0.13 at pH 1.2 and pH 7.4, respectively) indicating to drug diffusion through highly porous system. Formulations with high percent of APTES were unable to control the 5-FU release in time.

The presence of -amino groups at the surface of APTES particles affected the intensity of interaction with cell membranes and lead to increased muco/bioadhesive potential relative to the unmodified TEOS particles (Djurdjic et al., 2011). Percent of mucoadhesion for organically modified microparticles increased with increased proportion of APTES (55.7% of mucoadhesion for MP3).

Conclusion

The addition of precursor APTES modifies the morphology of silica microparticles, resulting in increased particle size, drug loading efficiency, muco/bioadhesive potential and 5-FU release rate compared to TEOS based particles. Mucoadhesive potential, burst release and dissolution rate for organically modified particles varied accordingly to the percent of APTES.

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Poster presentations

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Short communication

Doxycycline hyclate-enriched gelatine nanoparticles for periodontal disease treatment: preparation and evaluation study

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Introduction

Periodontal disease is chronic inflammatory disorder, believed to be generally caused by pathogenic microorganisms colonising the toot surface, amongst which the most popular are *Actonobacilius actinomyycetem comitans, Bacteroides forsitus, Porphyromonas gingivalis*, etc., being ~75% GRAM- anaerobic type. This disease affect the structural organs supporting the teeth, causing gingiva detachment from the tooth and periodontal pockets formation as an ideal ecological niche for the bacteria proliferation. Doxycycline (DOXY) is a wide spectrum antibacterial, bacteriostatic drug, effective against the aerob-/anaerobic type of GRAM+ and GRAM- bacteria as well as protozoa. It is a member of the tetracycline group, which is frequently used in dental treatments due to matrix metallo-proteinase- inhibitory effect and strong activity against periodontal pathogens (Tamimi et al., 2008). DOXY is almost completely absorbed in duodenum with a bioavailability of more than 80% with an average of 95%, and half-life of absorption is 0.85±0.41h.

Various drug delivery systems have been trailed in periodontitis treatments, such as fibres, gels, injectable systems, micro-spheres/particles, strips, compacts, films, and nanoparticles (NPs) (Raheja et al., 2013). Use of the later was found advantageous over the others due to their size allowing penetration in extra- and intracellular areas, such as gingival fluid, bacterial cells, from the gingival sulcus to the underlying connective tissue and to periodontal pocket areas below the gum line, being otherwise hardly accessible by different systems (Segundo-Pinon et al., 2000). Variety of materials have been proposed for drug-NPs processing; the important biomimetic merits of parent biopolymer (the biocompatibility and presence of multiple functional moieties) makes the gelatin (GEL) NPs effective delivery vehicles to be applicable for diverse therapeutics (Khan and Schneider 2013).

By this respect, presented study examines the applicability of nanoprecipitation as preferable methodology for DOXY-enriched GNPs processing. The variations range of processing factors, being selected within primary, trial-error experiments (i.e. the pH of GEL solution, DOXY concentration and EDC/NHS concentration) were established with utilization of ″one factor each time″ experimental approach. The limiting factor in all cases was drug/biopolymer precipitation. The influence of processing and formulation factors upon the particle size, drug content and encapsulation efficiency were estimate for processed formulation using MODDE software- generated experimental design. Chemical modification (crosslinking degree) and (in vitro) dissolution behaviour of selected DOXY-GNP formulation will be also discussed in this paper.

Materials and methods

Materials. GEL type B from bovine skin Bloom 225 (Sigma Aldrich, Germany), Doxycicline hyclate (DOXI) (Tocris, UK), Lutrol F-127 was kindly donated by BASF, Germany. 1-ethyl-3-(-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Sigma Aldrich, Germany. All other chemicals were of analytical grade and were used as received, without additional purification.

GNPs preparation. 0.5% w/v GEL/H2O solutions were prepared at 50°C under moderate stirring for 1h. The

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two separate solutions were prepared in parallel, and subsequently adjusted to pH 7.5 and pH 9.5. 1mL of selected solution was added dropwise to 10mL of 7% w/v Pluronic F-127 solution in 96% EtOH, latter being vigorously stirred onto magnetic stirred with 700rpm. Additional 10mL EtOH were added afterwards, just prior addition of different concentrations of cross linkers (20-25% w/w, relative to gelatin), being dissolved in 1.250 mL deionised water just prior experiments. One set of samples were removed from the magnetic stirred, while the other half were subjected to mild stirring for 24h. In parallel with DOXY-free HNPs, the DOXY-loaded one slight modification of above procedure, by simple mixing of pre- prepared DOXY and GEL solutions in quantities described within experimental design.

Characterization. Scanning Electron Microscopy / SEM/ imaging and Differential Light Scattering /DLS/ analysis were performed to evaluate particle size, PDI and aggregation behaviour in different physiological media and particle morphology. Drug content (%), encapsulation efficiency (%) and drug release (%) were quantified by optimised HPLC method (according to European Pharmacopoeia 5.0). Uv-Vis Spectroscopy (using tri nitro-benzene-sulfonic acid /TNBS/ reagent) and Attenuated Total Reflectance - Fourier Transform Infrared Spectroscopy / ATR-FTIR/ method were used to identify the crosslinking efficiency. Dissolution test was performed in relevant (saliva-like) conditions (phosphate buffer pH 7.4, 37°C, horizontal mixing) using cellulose ester- based Float a Lyser device (MWCO 100kDa) (at particular time point samples were withdrawn and analysed by HPLC means and Uv-detection /254nm/ of DOXY component).

Results and discussion

Physiologically stable drug-free, as well as DOXY-enriched GNPs were successfully processed by optimised nanoprecipitation method, where EDC/NHS chemistry was introduced in final processing phase. This chemical stabilization results in up to 70% crosslinking degree, identified by means of NH_2 group's reduction. Due to the presence of suitable chemical moieties and their respective pKa values, we assume that cross-linking occur not only between GEL molecules, but also between GEL and DOXY, and potentially, between the GEL and the Pluronic F-127. DOXI chemical ″arrestment″ was found to affect its dissolution profile, which, for particular formulation was estimate to 35% in 24 h period. Moreover, we observe significant effect of processing conditions, i.e. the mixing of DOXY-GNPs dispersions after nanoprecipitation influence on their aggregation behaviour, giving a clue for more complex interactions within examined, multicomponent system.

Resulting DOXY- integrated GNPs where visualised mainly as individual, spherically shaped NPs in 150-240 nm size range, while, relatively high encapsulation efficiency (up to ~40%) and drug content (25%) were identified for selected factors combination.

Conclusion

DOXY-enriched GNPs may be further applied as single formulation or included in other delivery system (e.g. Guided Tissue/Bone Regeneration /GTR, GBR/ membrane) and used in periodontal disease treatment. Nanoprecipitation methodology can be further explored in processing of GNPs as carriers of different types of drugs, thus serving as a processing platform for facile, yet controlled engineering of drug delivery carriers.

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Short communication

Prospective of PET radiopharmaceutical development –new approach and strategy for their application

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Introduction

The University Institute for Positron Emission Tomography of the Republic of Macedonia is the first center in our country with opportunity for production of PET radiopharmaceuticals, which are prepared as sterile solutions for parenteral use. In the first line of production are included the most used radiopharmaceuticals for PET imaging, such as: [18F] FDG; Na18F; [13N] NH3; [11C] Choline and 68Ga labelled somatostatin analogs.

Pharmacological approach of the PET radiopharmaceutical drugs – first line of production of The University Institute for PET of Republic of Macedonia

[18F] FDG

[18F] FDG is the most widely used PET radiopharmaceutical in clinical practice, especially in oncology, neurology and cardiology, providing functional information based on tissue metabolism. This radiopharmaceutical is a structural analogue of 2-deoxy-D-glucose labelled with positron-emitting isotope 189F in the position 2 of the glucose core structure. Widespread use of [18F] FDG is based on the principle of 'metabolic trapping' in the cancer cells.

After intravenous administration, [18F] FDG through the bloodstream is distributed into cells with the same mechanism as plasma glucose. It is actively transported into the cell mediated by a group of structurally related glucose transport proteins (GLUT). Tumor cells have an overexpression of GLUT compared to a normal cell, and therefore the uptake of [18F] FDG is increased. When the [18F] FDG is transported in the cells, it is phosphorylated in [18F] FDG-6-phosphate under the catalytic action of hexokinase and it remains metabolically trapped intracellularly, because [18F] FDG-6-phosphate is not a substrate for glucose-6-phosphate isomerase, the enzyme that metabolizes glucose (Scott et al., 2012).

The accumulation of [18F] FDG in the malignant cells generally is proportional to the metabolic activity of the cancer cells, which enables their detection by the PET scanner. [18F] FDG is non-specific cancer radiopharmaceutical. Increased accumulation of [18F] FDG occurs in processes such as inflammation, infection, especially sarcoidosis, tuberculosis, fungal infections, and pneumonia (Jacobson et al., 2012).

Normally increased accumulation of [18F] FDG is observed in the brain because the glucose is the main energy source for the brain. Also very important is the concentration of glucose in the blood of the patient and it should be evaluated prior to administration of [18F] FDG. The increased concentration of blood glucose causes elevated levels of insulin, which increases biodistribution of [18F] FDG in muscle and adipose tissue (Lindholm et al., 1993).

[18F] Na18F

[18F] Na18F PET/CT is diagnostic tool for imaging benign and malignant bone diseases. Fluoride ions are incorporated into the bone matrix at the bone surface preferably in sites of newly mineralizing bone, such as during growth, infection, malignancy (primary or secondary), after trauma or during inflammation.

The initial [18F] Na18F distribution represents blood flow that varies among different bones. Almost all deliv-

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ered [18F] Na18F is retained by bone after a single pass of blood. The uptake of fluoride ions is better in osteoblastic processes, while purely osteolytic processes have a lower uptake or even no uptake at all. [18F] Na18F is rapidly cleared from plasma and excreted by the kidneys. The target organ is bone, but approximately 20% is excreted through the kidney in the urine in the first 1-2 hours (Czernin et al., 2010).

[13N] NH3

The main clinical application of [13N] NH3 radiopharmaceutical is assessment of myocardial perfusion in patients with suspected or known coronary heart disease.

Following intravenous injection, [13N] NH3 rapidly clears from the circulation. It is taken up mainly by the myocardium, brain, liver, kidneys, and skeletal muscle. [13N] NH3 is extracted from the capillaries through the ammonia transporter. The accumulation in tissue is in proportion to blood perfusion of the tissue.

In the cells, it is converted to glutamine and can diffuse out of the cell or be metabolized to glutamate and retained within the cell. [13N] NH3 undergoes a five-enzyme step metabolism in the liver to yield [13N] Urea, the main circulating metabolite, which is eliminated from the body by the urinary excretion (Adeva et al., 2012).

[11C] Choline

[11C] Choline is radiopharmaceutical for oncological PET imaging of tumors which overexpress choline kinase. Result of overexpression of choline kinase is increased level of phosphorylcholine by accumulation of free choline for cell membrane synthesis. [11C] Choline is accumulated preferentially within prostate cancer tissue, for that reason the most important application of this radiopharmaceutical in clinical practice is in visualization of this type of tumor. [18F] FDG is not first choice in visualization of the tumors in pelvis area, because of the low uptake, related to lower expression of glucose transport proteins and to the huge [18F] FDG urinary excretion (Lodi et al., 2012).

The maximal tumoral[11C] Choline uptake is related to primary tumor stage (Reske et al., 2006).After intravenous administration, the peak of uptake is reached by five minutes and the activity is retained over the subsequent 30 minute scanning period. The distribution of [11C] Choline is mainly to the pancreas, kidneys, liver, spleen and colon. The major metabolite detected in blood is [11C] Betaine (Roivainen et al., 2000).

68Ga labelled somatostatin analogs: [68Ga] DOTATOC, [68Ga] DOTANOC and [68Ga] DOTATATE

The use of [68Ga] DOTATOC, [68Ga] DOTANOC and [68Ga] DOTATATE radiopharmaceuticals for visualization of neuroendocrine tumors is based on high affinity of the biological ligand to somatostatin receptors (SSTR). Natural somatostatin has low metabolic stability, therefore synthetic analogues with high affinity for SSTR and resistant to enzymatic degradation are developed. (Velykian, 2014).

[68Ga] DOTA SST analogues show a rapid localization of the target site, fast blood and renal clearance. Radioactive metabolites are not detected in serum or urine after 4 hours. The maximum accumulation of activity in the tumor is reached 70 ± 20 min after injection. Excretion is almost entirely by the kidneys.

Conclusion

The mapping of the radiopharmaceutical distribution in vivo provides images of functional morphology of organs in a non-invasive manner and plays an important role in the diagnosis of many common diseases associated with the malfunctioning of organs in the body as well as in the detection of certain type of cancers (IAEA, 2006).

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Short communication

Cosmetovigilance

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Definition of a cosmetic

The human need for improvement of the physical appearance is in constant increase year after year. This is revealed by various types of tests and ongoing market research. Hence, the production, the application and the consumption of products for personal hygiene as well as cosmetic products for care, protection and enhancement of the beauty are becoming more and more popular among large number of consumers.

A cosmetic product is classically defined as any preparation that is applied to the skin, eyes, mouth, hair or nails for the purpose of cleansing, appearance enhancement, protection and/or for pleasant smell. Unlike drugs, which are used for treatment or prevention of a disease in the body, cosmetics are not considered to change or affect the body's structure or functions. However, the distinction between drugs and cosmetics is sometimes not well defined. In spite of the considerable safety of and the high skin tolerance for the cosmetic products, adverse effects still occur from their use, which might be a result from the use of an inappropriately chosen product. Most often these adverse effects are of small or medium intensity and appear usually on the skin (Evaluation of the Cosmetics and Explosives Directives, 2007; Regulation (EC) N0 1223/2009). The research subject of this work is cosmetovigilance, which refers to a series of defined actions that have the purpose of revealing, estimating, monitoring, recording and preventing the potential undesirable reactions that appear as a consequence of the application of a cosmetic product.

The concept of cosmetovigilance is recently added to the European Regulation for cosmetic products. It is a form for objective monitoring of the unwanted effects of the cosmetic products, which are of public health interest. In contrast to the monitoring carried out by the industry, the aim of which is the safety of its own market for commercial purposes, cosmetovigilance has strictly medical aim (Vigan and Castelain, 2014).

The European regulation

The European resolution (ResAP (2006)1) from 2006 laid the foundations of the cosmetovigilance system which is based on the reports from the recorded cases. The European Regulation (EU) 1223/2009 for cosmetic products, which was officially introduced on 11 July 2013, requires from all manufacturers to appoint a responsible person who would constantly monitor and record the implementation of the cosmetovigilance for the products available on the market. From 2013 onwards, the new European regulation demands that the serious undesirable effects which are reported to the competent authority to be also delivered to the competent authority of the other member states as well as to the responsible person for a certain cosmetic product in the company. The regulation for cosmetic products primarily addresses the safety of the products that are used by large populations of healthy consumers. However, the efficacy and the safety of cosmetic products are not reviewed or approved by national competent authorities before they are sold to the public. The identification and the analysis of the adverse effects related to cosmetic products is a process that is still to a large extent industry-driven. It is a manufacturers' responsibility to determine that the product and its ingredients are safe before they are put on the market. Moreover, manufacturers have the responsibility of collecting the reports for all the recorded adverse effects. Although manufacturers do their best to ensure safety of their products, it should be taken into consideration that a potential conflict of interests is always present (Moretti and Velo, 2008).

The aim of this work is to present the implementation of the cosmetovigilance system in the countries in Europe

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and how it is employed in practice. Also this work would attempt to resolve two major problems: evaluation of the causes and the types of reports. At the same time it would present data gathered from conducted studies the aim of which was to estimate the appearance of adverse effects from the use of a cosmetic product, utilizing different methods for their detection, monitoring and prevention, as well as establishing a standard cosmetovigilance system.

Causality assessment

The definition of causality assessment is slightly different for French Health Products Safety Agency (AFSSAPS) and European Cosmetic, Toiletry and Perfumery association (Colipa). For AFSSAPS, causality "assesses the cause and effect relationship between a cosmetic product and a specific clinical and/or paraclinical manifestation" (2010). Causality must be established for each product individually. For Colipa "Causality assessment is particularly useful when the same product is involved in the occurrence of several cases of undesirable effects, when it makes it possible to determine the extent of a link of cause and effect between the cosmetic product and the undesirable effects observed and then to take these effects into account in the subsequent drawing-up of corrective measures such as investigations, recommendations on the proper use of the product, or regulations at national or European level (restrictions on use, warnings on packaging labels, limited concentration or prohibition) (Zweers et al., 2012).

Several methods have been published. They are based on the analysis of evolving chronological and semiological elements. The results of relevant tests or of re-challenge tests can alter causality, for instance as regards contact allergy; appropriate patch testing provides a certain degree of causality. The AFSSAPS method is based on 6 criteria, divided into two groups, which are used to calculate a chronological score and a semiological score. The level of causality is determined using a decision table in which the scores are combined. The method has five levels of causality assessment: very likely, likely, not clearly attributable, unlikely and excluded. The causality assessment method by Colipa is based on three major criteria: symptomatology, chronology and results of specific tests. This method offers 3 levels of causality on the basis of a decision tree in which these criteria are combined: questionable, likely and very likely. Another method uses a flow chart as soon as the case has been reported, following a PLM (product lifecycle management) call approach. It is also based on chronological and semiological criteria and all the notifications can be analysed using 6 levels: irrelevant, not enough information, unlikely, possible, probable and certain. (The SCCPS notes of guidance for the testing of cosmetic ingredients and their safety evaluation, 2006).

The cosmetovigilance system is the right means of obtaining information on the safety of cosmetic products and their ingredients. It can be used by the competent authorities in Europe, which would confirm that the new directives ensure a high level of safety. Cosmetovigilance makes it possible to exclude and control potentially hazardous ingredients which are included in the product content, and thus develops the costumers' trust and reassures them about the safety of the products available on the market.

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Good Distribution Practice for medicinal products

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Good distribution practice (GDP), as part of quality assurance system, for medicinal products for human use includes myriad requirements for purchase, receiving, storage and export of drugs intended for human consumption. GDP regulates the division and movement of pharmaceutical products from the premises of the manufacturer of medicinal products, or another central point, to the end user thereof, or to an intermediate point by means of various transport methods, via various storage and/or health establishments.

The wholesale distribution of medicinal products is an important activity in integrated supply chain management. Today's distribution network for medicinal products is increasingly complex and involves a lot of people from different professional profiles.

The GDP Guidelines establish appropriate tools to assist wholesale distributors in conducting their activities and to prevent falsified medicines from entering the legal supply chain. Compliance with these Guidelines will ensure control of the distribution chain and consequently maintain the quality and the integrity of medicinal products (GGDP, 2103).

The GDP Guidelines are intended to be applicable to all persons and outlets involved in any aspect of the storage and distribution of medicines from the premises of the manufacturer of the product to the person dispensing or providing medicines directly to a patient or his/her agent. This includes all parties involved in trade and distribution of medicines, including the manufacturers of bulk, finished products, wholesalers, as well as others such as suppliers, distributors, Government institutions, international procurement organization, logistic providers, traders, transport companies and forwarding agents and their employees as well as health workers. The main parts of GDP are: 1) Organization and management; 2) Quality management; 3) Personnel; 4) Equipment and premises; 5) Documentation; 6) Operations; 7) Outsourced activities; 8) Self inspections and 9) Transportation. In fact, the parts mentioned above form the GDP network. They interfere between each other forming a complex system in order to provide quality assurance that ensures that the quality of pharmaceutical products is maintained through adequate control throughout the numerous activities which occur during the distribution process. Every part of the GDP system is important to maintain the quality of the distribution chain. Regarding Organization and management, an adequate organizational structure for each entity in the chain of distribution should be defined with the aid of an organizational chart. The aim of this organizational chart is to have a clear view of the duties and responsibilities of each person involved in the process (GGDP, 2103; WHO, GDP, 2005).

Every individual involved in the process of distribution should have a written job description and at every level of the supply chain, employees should be fully informed and trained in their duties and responsibilities.

Training should be based on written standard operating procedures (SOPs). Personnel should receive initial and continuing training relevant to their tasks, and be assessed as applicable, in accordance with a written training program.

The system for managing quality should encompass the organisational structure, procedures, processes and resources, as well as activities necessary to ensure confidence that the product delivered maintains its quality and integrity and remains within the legal supply chain during storage and/or transportation. The quality system should be fully documented and its effectiveness monitored. All quality-system-related activities should be defined and documented. A quality manual or equivalent documentation approach should be established. A responsible person should

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be appointed by the management, who should have clearly specified authority and responsibility for ensuring that a quality system is implemented and maintained.

This responsible person is designated by the wholesale distributor and should meet the qualifications and all conditions provided for by the legislation of the Member State concerned. A degree in pharmacy is desirable. The responsible person should have appropriate competence and experience as well as knowledge of and training in GDP (GGDP, 2103; GGDPBP, 2012).

Besides the responsible person there should be an adequate number of competent personnel involved in all stages of the wholesale distribution activities of medicinal products. The number of personnel required will depend on the volume and scope of activities.

Wholesale distributors must have suitable and adequate premises, installations and equipment, so as to ensure proper storage and distribution of medicinal products. In particular, the premises should be clean, dry and maintained within acceptable temperature limits.

Different medicines require different storage conditions. There are medicines that should be stored following the cold chain regime and medicines that should be stored on room temperature, 2-8 °C and 15 -25 °C respectively (Commission Directive 2003/94/EC, 2003; WHO, GDP, 2005).

Equipment and processes should be respectively qualified and/or validated before commencing use and after any significant changes, e.g. repair or maintenance.

There are a lot of SOPs (standard operative procedures) that determine every step of the processes that are conducted during the reception, storage and dispatch of the medicines.

All actions taken by wholesale distributors should ensure that the identity of the medicinal product is not lost and that the wholesale distribution of medicinal products is performed according to the information on the outer packaging. The wholesale distributor should use all means available to minimise the risk of falsified medicinal products entering the legal supply chain.

Also in compliance with the GDP standard qualification of the supplier and costumers must be done.

Regarding the situations that can occur after the patient has received the medicines, there must also be a SOP on how to handle a complaint from the costumer as well as a SOP for medicines that are returned for some reason.

In order to provide a continuous quality management system, self-inspections must be conducted in an impartial and detailed way by designated competent company personnel. Audits by independent external experts may also be useful but may not be used as a substitute for self-inspection.

The transportation of the medicines is a very important part of the distribution process, that is why the personnel in charge of transportation must be trained how to operate with the medicines that should be distributed and the vehicles involved in the process must be suitably calibrated and mapped as well.

Considering all the facts mentioned above a conclusion can be made that the Good Distribution Practice (GDP) system is a very important tool in order to provide a better medicine quality regarding distribution factors and an improved health care system as well, which will contribute for a higher safety of the patients (Commission Directive 2003/94/EC, 2003; GGDP, 2103).

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