Application of the QbD-based approach in the early development of liposomes for nasal administration

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Abstract

In this study, authors adapt the Quality by Design (QbD) concept as well as the Risk Assessment (RA) method to the early development phase of a new nano-sized liposomal formulation for nasal administration with brain target. As a model active agent, a BCS II class drug was chosen to investigate the behaviour of the drugs with lipophilic character. This research presents how to apply this risk-focused approach and concentrates on the first four stages of the QbD implementation. In this way the quality target product profile was defined, the critical factors were identified and an RA was performed. The RA results helped in the factorial design-based liposome preparation by the lipid film hydration method. The prepared liposomes were evaluated (vesicle size, size distribution, and specific surface area). The surface characteristics were also investigated to verify the exactness of the RA and critical factors based theoretical prediction.

The results confirm that the QbD approach in liposome development can improve the formulation process. The RA focused predictive approach resulted in a decreased number of studies in practice but in an effective product preparation. Using such innovative design and development models can help to optimise and rationalise the development of liposomes.

Keywords: Quality by Design; Risk-based formulation; Risk assessment; Liposome; Lipophilic drug containing liposome; Nasal administration; Nanocarriers

Abbreviations: API, active pharmaceutical ingredient; BCS, Biopharmaceutics Classification System; CMAs, Critical Material Attributes; CNS, central nervous system; CPPs, Critical Process Parameters; CQAs, Critical Quality Attributes; D[3,2], surface weighted mean; D[4,2], volume weighted mean; DoE, Design of Experiments; PdI, polydispersity index; Ph Eur, European Pharmacopoeia; QTPP, Quality Target Product Profile; RA, Risk Assessment; RPN, risk priority number; rpm, rotation per minute; SEM, scanning electron microscope; SSA, specific surface area; WF1, wall-forming agent 1 (in this study: natural oil/cholesterol); WF2, wall-forming agent 2 (in this study: Phospholipon 90G®)

1 Introduction

The Quality by Design (QbD) is a risk- and knowledge-based quality management approach of the pharmaceutical formulation development described in ICH guidelines (Ich. Pharmaceutical, 2009; Türker et al., 2004), which starts with the expected quality and therapeutic goals. In this case, the product and the manufacturing process are designed and developed according to the previously defined expectations, which means a change in paradigm (Yu, 2008; Yu et al., 2014). A complete QbD study involves the following stages (Ich. Pharmaceutical, 2009): (1) Define the Quality Target Product Profile (QTPP) based on the knowledge space developed from the relevant scientific literature and with appropriate in vivo relevance. (2) Design the product and the manufacturing process in accordance with the predefined profile. (3) Identify the potential Critical Quality Attributes (CQAs), the potential Critical Material Attributes (CMAs) as well as the potential Critical Process Parameters (CPPs), and perform the Risk Assessment (RA) (Quality Risk, 2005), which is the key element of the QbD approach.

(4) Set up the Design of Experiments (DoE) based on the RA results. DoE is a practical development planned and executed according to the most relevant influencing factors (CQAs, CPPs) selected by the priority ranking of the RA in order to define the Design Space. (5) Set up a process control strategy to ensure consistent product quality. (6) In the industry, the last step is the product lifecycle management. Overall, the application of the QbD concept results in a better product, process, and interrelation understanding (Patil and Pethe, 2013). The QbD-indicated theoretical prediction and the risk-focused approach help to conduct a more effective practical research activity, which is

adaptable even in the early phases of pharmaceutical developments (Pallagi et al., 2015; Gieszinger et al., 2017; Pallagi and Karimi, 2016; Karimi and Pallagi, 2016; Csóka et al., 2018).

Liposomes- as nano-carriers have many (Akbarzadeh et al., 2013). They are artificial spherical particles with an aqueous core surrounded by a hydrophobic membrane, which forms a special lipid bilayer structure and has an amphiphilic character (Deamer, 2010), thus they are able to enclose both lipophilic (located in the nonpolar wall) and hydrophilic compounds (placed in the aqueous polar inner phase) into their structure (Laouini et al., 2012). The encapsulation of the molecules protects the active agents and allows better targeting (Torchilin, 2007; Maherani et al., 2011). Lecithin or other phospholipids (natural or artificial) and cholesterol are the main components of liposomes (Briuglia et al., 2015). The bilayer structure is formed from phospholipids in an aqueous milieu. Its rigidity and the permeability depend on the length and the saturation of the carbon chains. The release of the active agents of liposomes is modified by the phase transition temperature of the lipid compound. Cholesterol builds into the membrane and decreases the fluidity of it (Briuglia et al., 2015). By surface modification (eg Polyethylene glycol chains) the in vivo circulation time can be increased (Torchilin, 2007), so the therapeutic index can be raised and the toxicity can be reduced. Usually, liposomes as carriers are used in the case of drugs with low permeability (according to the Biopharmaceutics Classification System (BCS) these belong to BCS classes III and IV Rodriguez-aller et al., 2015; Zylberberg et al., 2016; Akbarzadeh et al., 2013). The drugs which exhibit high permeability (BCS Class II and Class IIb weak bases) may precipitate (in a very complex and poorly understood method) in the small intestine (Tubic-grozdanis et al., 2008). The intestinal precipitation of a Class IIb drug can depend on numerous factors (e.g. formulation, physiological factors, time of administration, etc.) and complicates in vivo prediction (Tsume et al., 2014). The incorporation (entrapment) of lipophilic BCS II drugs into liposomes and the choice of an alternative administration route like the nasal administration can offer several advantages in the therapy. Compared to the conventional administration routes (Allen et al., 1993) (e.g. intravenous administration, dermal or pulmonary intake), *nasal administration* is a non-invasive, simple and painless manner of drug delivery, able to reach an immediate and local effect, but also to get a long-term and systemic therapeutic effect (Türker et al., 2004). The active agent administered into the nasal cavity can be absorbed into the systemic blood circulation, reach the central nervous system via the blood-brain barrier (“nose-to-blood”), or reach the brain tissue directly by axonal transport. This is the so-called “nose-to-brain” route, which avoids the blood-brain barrier and results in the targeted delivery of the active pharmaceutical ingredient (API) into the central nervous system (CNS) from the nasal cavity (Bartos et al., 2018) which may help in the treatment of Alzheimer’s and Parkinson’s disease or epilepsy in the future (Agarwal et al., 2013). However, the “nose-to-brain” way has several limitations. Water-soluble materials and molecules with molecular weights higher than 40-500 Da have no, or only very limited potential to pass the blood-brain barrier, while hydrophilic and polar molecules have several limitations in the paracellular transport between the epithelial cells (Bartos et al., 2018). Lipophilic molecules can pass easily via the transcellular pathway by passive diffusion. Further general characteristics of nasal delivery are the following: Successful formulation and the therapeutic effect depend on molecular weight and size, solubility, lipophilic characteristic, the pKa value and the distribution coefficient of the API because these features influence absorption. The nasal absorption of the API depends on pH. In general, better absorption can be achieved if the pH of the API is lower than its pKa value. The normal physiologic pH is 5.5-6.5 in the nasal region, thus the product should have a pH value of 4.5-6.5. The volume of the nasal cavity is limited, so only a small amount but a highly concentrated product can be administered there (200–300 µl pro dosi) (Bartos et al., 2015). The mucus on the nasal mucosa is renewed in every 15–20 min due to the continuous mucus secretion and mucociliary activity. So, if the bioadhesivity of the product is poor, the half-life of the API is 10-15 minutes. That can be solved by using mucoadhesive excipients. Better solubility and permeability values can be expected from nanosized particles (or nanosized drops) The the optimal particle size range for nasal administration is 200–500 nm and 100–400 nm if the target is specifically the nose-to-brain route (Sonvico et al., 2018, 2018).

This work was aimed to adapt the QbD as well as Risk Assessment to the early development phase of a new liposomal formulation for brain target with nasal administration. A lipophilic drug (lamotrigine) was chosen as a model active agent in order to study the incorporation of drugs with high permeability into the liposomes, and investigate the potential advantages of this process. This research focuses on the first four stages of QbD implementation, so the aimed tasks were to define the target profile, select the critical factors, perform a Risk Assessment and a factorial design-based liposome preparation, and evaluate the results in order to verify whether the QbD approach in a liposome development can improve the formulation process and can help to make it more effective and efficient.

2 Materials and methods

2.1 Materials

The applied model API was a lipophilic (BCS Class II) drug with low solubility and high permeability properties (lamotrigine, $C_9H_7Cl_2N_2$, Meditop Pharmaceutical Ltd., Pilisborosjenő, Hungary). The molar weight of the API is 256.09 g/mol, the melting range is 216–218 °C, the solubility is 170 mg/L in water and 12 mg/L in ethanol at 25 °C, the pKa is 5.7, and the value of the logP is 2.5 measured in distilled water.

The following materials were used as excipients: Phospholipon 90G® lipid mixture (Phospholipid GmbH, Köln, Germany), cholesterol (in an alcoholic solution) (Molar Chemicals Ltd., Budapest, Hungary), natural organic apricot kernel oil (Primavera Life GmbH, Oy-Mittelberg, Germany) as components of the liposomal wall, ethanol 96% (Molar Chemicals Ltd., Budapest, Hungary, official in the Ph. Eur.) was chosen from “green chemistry” considerations (Green, 2018; Bacher, 2014), as a relatively nontoxic solvent and sodium chloride physiological solution (NaCl, Molar Chemicals Ltd., Budapest, Hungary). The excipients were used for different purposes and in different ratios according to the sample preparation process (Table 1).

Table 1 Compositions of the prepared API-containing liposome samples

Preparation process number	Concentration of phospholipid (Phospholipon 90G®)	Temperature of the ultrasound treatment	Number of the (second) filtration	Samples prepared E = Empty A = API containing
1	95	65	1	E-95-1-65 A-95-1-65
2	95	55	1	E-95-1-55 A-95-1-55
3	85	65	1	E-85-1-65 A-85-1-65
4	85	55	1	A-85-1-55 E-85-1-55
5	95	65	3	E-95-3-65 A-95-3-65
6	95	55	3	E-95-3-55 A-95-3-55
7	85	65	3	E-85-3-65 A-85-3-65
8	85	55	3	E-85-3-55 A-85-3-55

The amount of cholesterol, oil component and API (in the API containing samples) were constant in each preparation.

2.2 Methods

2.2.1 Definition of the QTPPs and knowledge space development

The initial step is to define the target product profile and its quality criteria. The selection of the QTPPs is based on the requirements of the interested parties (clinical expectations, patients' and industrial needs, regulatory aspects). It usually includes the route of the administration, the dosage form, the dose, the appearance, and the dissolution or the pharmacokinetic data of the drug, furthermore, some special safety or quality requirements, etc. In order to see all the influential parameters of the desired liposome product, a cause and effect (Ishikawa) diagram Tague (2005) was constructed to collect and visualize these factors for the further steps. This diagram is especially useful for selecting the QTPPs and the critical factors during the next QbD-guided development steps.

2.2.2 Determination of the CQAs

The second step is the selection of the factors which have critical effects on the targeted product quality. These are the Critical Quality Attributes. The selection of these parameters is based on prior knowledge and previous experience. Usually, CQAs are physical, chemical, biological or microbiological properties that should reach an appropriate range or limit to ensure constant end product quality. CQAs may include information about particle or vesicle size, size distribution, drug release, etc. The selection of a factor as a CQA always depends on the predefined goals, the expected quality of the product and the therapeutic needs.

2.2.3 Determination of the CMAs and the CPPs

In the next step, the critical material and/or the Critical Process Parameters should be determined, which are the factors relating to the materials and the selected production methods and processes, thus may influence the CQAs.

2.2.4 Initial Risk Assessment

After the identification of the QTPPs, the CQAs, and the CMAs/CPPs, an initial RA was carried out via the following steps: interdependence estimation between the QTPPs and the CQAs and between the CQAs and the CMAs/CPPs using a three-grade scale, whether the estimated interdependence is high, medium or low. The next task is the estimation of the occurrence of the CPPs/CMAs, using the same scale. RA was performed through the Lean QbD Software® (QbD Works LLC, Fremont, USA). According to the calculation of the software, all of the critical factors were quantified and ranked by their influence. The rankings of the CQAs and CPPs were visualized on Pareto charts Powell et al. (2014) generated by the software. The Pareto charts not only show the relationships between the CMAs or the CPPs and the CQAs but also help to select the factorial design parameters of the experiments.

2.2.5 Design of experiments

For the design of the practical experiments, the JMP® 13 Software (SAS Institute, Cary, USA) was used and the Main Effect Screening Design 2 programme was chosen. For this screening design, the variable factors were selected based on the RA results. These were the concentration of phospholipids (X1, lower limit: 85 w/w%, upper limit: 95 w/w%), the temperature of evaporation during the lipid film formation (X2, lower limit: 55 °C, upper limit: 65 °C) and the number of the second filtration with the 0.22-µm membrane filter (X3, lower limit: 1 filtration, upper limit: 3 filtrations). For a response, the size and the size distribution of the vesicles were investigated. The pattern of the factorial design was the following (X1, X2, X3, – is the lower limit and + is the upper limit), sample 1: ++-, sample 2: +--, sample 3: -+-, sample 4: ---, sample 5: +++, sample 6: +-+, sample 7: -++, sample 8: --+. The same pattern was used during the preparation of API-containing and API-free liposomes as well.

2.2.6 Preparation of liposomes

The liposomes were prepared by using the lipid film hydration method (also called as thin film method) (Dua and Bhandari, 2012). The first step was the preparation of the lipid suspension, which was a mixture of the established amount of Phospholipon 90G®, cholesterol, and natural oil. API-free (empty) and API-containing liposomes were prepared (Table 1). The preparation was based on the pattern of the factorial design. In the case of the API-containing product, the defined amount of API (250 mg) was added to the lipid phase. 50 ml of ethanol 96% was used as a solvent agent. Ethanol was evaporated under vacuum, in a water bath, with Rotavapor® R-210/215 (BÜCHI Labortechnik AG, Flawil, Switzerland), at 55°C or 65°C. The rotation speed was 25 rpm. The vacuum creation was gradual (from 1100 mbar to 300 mbar, with steps of 100 mbar, from 300 mbar to 150 mbar with steps of 50 mbar). Then, the dry lipid film was hydrated with 3x50 ml of physiological saline solution. Hydration was supported by ultrasonication (Elma Transsonic Digital S D-78224 ultrasonic bath, Elma Schmidbauer GmbH, Singen, Germany) at 55°C or 65°C, 120% power, 48 kHz, for 15 minutes. The shaping of the liposomes was performed in two steps. First, a 0.45-µm membrane-filter was used for one filtration (Millipore® SLHV033NS Millex® HV Syringe Filter with Durapore® PVDF Membrane, Sigma-Aldrich) with a 10-ml syringe, then a 0.22-µm membrane-filter for 1- or 3-time filtration (FilterBio PES Syringe, FilterBio Membrane Co. Ltd, Lab-Ex Labortrading Ltd.). This step was followed by freeze-drying (lyophilization) to stabilise the prepared samples and fit for the SEM investigation.

The preparation conditions of the liposome samples are presented in Table 1. Besides the API-free, empty samples API-containing formulations were also prepared, thus 16 liposome sample were prepared all together (Table 1).

2.2.7 Characterization of the liposomes

2.2.7.1 Particle size and zeta potential analysis The vesicle size of the prepared liposomes was determined by using two instrumental methods. The first was the laser diffraction (Mastersizer 2000, Malvern Instruments Ltd Worcestershire, UK) method. This technique was applied to determine the most commonly used units, the d-values, namely d(0.1), d(0.5) and d(0.9) values. These data describe particle size distribution and mean 10% undersize, 50% undersize (media diameter) and 90% undersize, respectively of the total values. The equipment calculates the span value as well. The span value provides the width of distributions (Rawle), namely span = (90% undersize − 10% undersize)/50% undersize. The higher span valu may indicate greater polydispersity of the system. The uniformity of the samples can also be measured from size distribution, as well as specific surface area (SSA), surface weighted mean (D[3,2]) and volume weighted mean (D[4,2]). These two values, D[3,2 and D[4,2], both mean an average of the particle size and have an important role in flow dynamics. The second instrument, which was used for the determination of particle size and zeta potential of some of the samples, was based on the dynamic ligh scattering technique (Malvern Zeta Nano ZS, Malvern Instruments Ltd, Worcestershire, UK, Refraction Index: 1.33, 25°C). This investigation resulted in the mean vesicle size, polydispersity index (PdI) values and the surface charge of the prepare samples.

2.2.7.2 Scanning electron microscopy (SEM) We used a Hitachi S-4700 FE-SEM scanning electron microscope (Hitachi High-Technologies Europe GmbH, Krefeld, Germany) for this process. The freeze-dried samples were coated wit gold-palladium by a sputter coater (Bio-Rad SC502; VG Microtech, Uckfield, UK). The used air pressure was 1.3–13.0 mPa. Freeze-drying (Van Winden et al., 1997) is necessary before the SEM investigation, it was made by Coolsafe 100-9 Pro ScanVa (LaboGene ApS, Lynge, Denmark, from 25 °C/normal pressure to −40 °C/0.01 atm under vacuum, after 8 hour the reheating steps were: −40 °C, −20 °C, 0 °C, +10 °C, +20 °C +30 °C until reaching the normal pressure).

Drug entrapment efficiency

The selected sample prepared by the lipid film hydration method was centrifuged with a Hermle Z 323 K centrifuge (Hermle LaborTechnik GmbH, Wehingen, Germany) for 10 minutes at 2240 rcf. 1 ml of 10-time dissolved samples was investigated and washed 2 times with sodium chloride physiological solution. The spectrophotometric investigation of the samples was conducted with a Unicam UV/VIS spectrophotometer (Unicam, Cambridge, UK) at a wavelength of 296 nm (r (Ich, 2009) = 0.998963, absorbance value = 0.0190 × concentration).

2.3 Statistical analysis

The statistical analysis of the results of the investigational data was performed with Microsoft® Excel® (Microsoft Office Professional Plus 2013, Microsoft Excel 15.0.5023.100, Microsoft Corporation, Washington, USA) and the JMP® 13 Software (SAS Institute, Cary USA).

3 Results and discussion

3.1 Definition of the QTPP, knowledge space development, identification of the CQAs, CMAs and CPPs

In the first step of this QbD-based liposome development, all the factors which could influence the aimed product were collected. This data collection process is knowledge space development. As a result, an Ishikawa diagram, namely a cause and effect diagram, was constructed from the collected factors, which is presented in Fig. 1. The influencing factors were classified into 4 groups, such as therapeutic aim, material characteristics, formulation technology, and product characteristics. All the mentioned items could have an impact on the quality of the lipophilic model drug-containing liposomes to be produced.

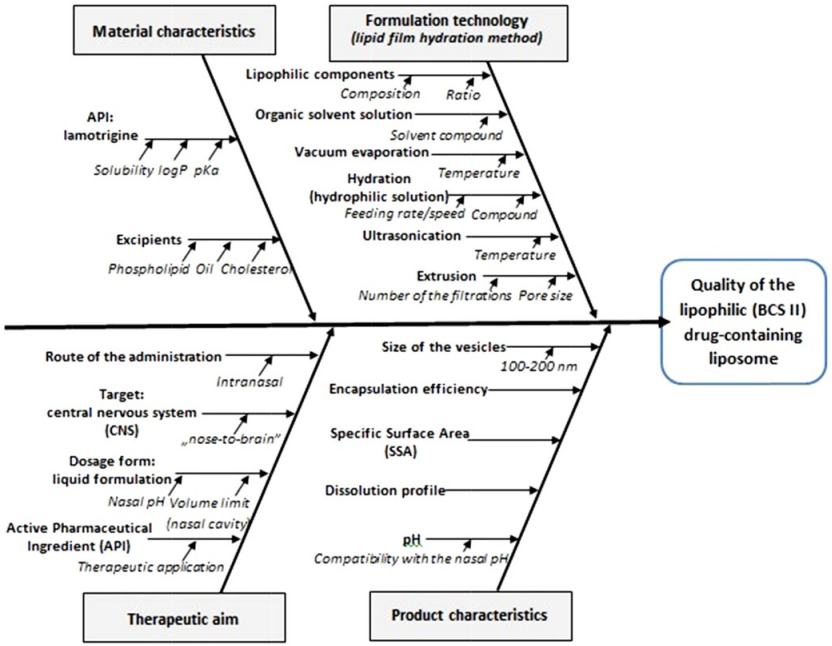


Fig. 1 Ishikawa diagram: it shows a cause and effect relationship between the material and process variables to specify the quality properties of the aimed liposomal product

After knowledge space development, the QTPP was defined. The predefined goal of this study was to develop a lipophilic drug-containing liposomal liquid formulation for nasal administration with brain target. Another aim was to prepare this nasally administrable target product in a proper volume and API dose, with adequate pH values, with an optimal dissolution and API release profile, and to reach the required stability of the product. These elements and the whole profile of this target liposomal product are presented in Table 2.

Table 2 Quality Target Product Profile (QTPP) of the aimed lipophilic drug-containing liposome product.

QTPP parameter	Target	Justification
Therapeutic effect	Reach the brain tissue (in the case of this model lipophilic API, the potential target is the antiepileptic effect)	Direct or axonal transport of the API from the nose to the brain instead of the route through the blood-brain barrier Nunes et al. (2012)
Patient group	Adults or even children	The chosen model API is safely administrable in both age groups Schlumberger et al. (1994)
Route of administration	Nasal route	The nasal route offers direct access to the CNS Serralheiro et al. (2015)The nasally applied single dose of the API can be decreased compared to the orally administered dose.
Dosage form	Liquid formulation	The usage of the liquid form of the liposomal formulation offers a comfortable method for the administration of the drug into the nasal cavity Illum (2003)
Volume of one dose	200–300 µl	The nasally administrable volume is limited by the capacity of the nasal cavity Pires and Santos, (2017). The volume of the preparation is linked to the efficacy of the formulation. It is necessary that the total volume of the formulation be enough to contain the optimal dose of the API
Dissolution	10–15 min	The dissolution profile of the product is related to the efficacy of the formulation. The proper amount of the active agent has to be dissolved in 10-15

profile/absorption time		minutes, which is the periodic renewing time of the nasal mucosa Hussain (1998), Arora et al. (2002).The characteristics of the optimal formulation fit to the conditions of the nasal cavity, which results in a controlled dissolution of the API
Stability (size of the vesicles)	100–400 nm	The size stability of the prepared liposomes is linked to the efficacy and quality of the preparation.The targeted size range is optimal according to the literature Sonvico et al. (2018)
pH	4.5–6.5	The optimal pH of the preparation fits to the normal pH of the nasal environment. The proper pH value ensures comfort during the application, and determines the quality and the in vivo efficacy of the product Parvathi (2012)

In the following step, according to the QbD-based formulation development, the Critical Quality Attributes (CQAs) were identified and selected. These factors have potential critical effects on the desired quality of the final product (Table 3).

Table 3 CQAs of the aimed liposome product, their target and justification.

CQA	Target	Justification
Wall-forming agent 1 (Natural oil/Cholesterol)	Optimal vesicle size	Natural oil and cholesterol influence membrane fluidity and the size of the vesicles Jójárt-Laczkovich et al. (2018) These components take part in the formation of the phospholipid bilayer Briuglia et al. (2015)
Wall-forming agent 2 (Phospholipon 90G®)	Optimal vesicle size	Phospholipon 90G® is a phospholipid complex and takes part in the formation of the phospholipid bilayer, furthermore, it influences the size of the vesicles Khale et al. (2011)
Hydration media	Optimal stability (no aggregation) of the liposomes	The hydration of the lipid film is one step of the formation of the phospholipid bilayer.
API	The successful incorporation of the lipophilic API into the liposomal wall	The incorporation of the API into the liposomal product could influence the size of the vesicles Jouyban and Soltanpour (2010)
Surface charge	Optimal for the liposomes to pass through the nasal membrane	The charge of the surface influences the stability of the vesicles, thus affects membrane transport Bozzuto and Molinari (2015)
Vesicle/particle size (and size distribution)	Around 200 nm	The size of the vesicles was chosen with regard to the aimed CNS target of the API Zawada (2004)
Stability of vesicle size	No aggregation, constant vesicle size	The stability of the vesicle size is important regarding the safety, the efficacy and the quality of the product Ich. Pharmaceutical, (2009), Winterhalter and Lasic (1993)

Parallel to the identification of the CQAs, the type of the liposome production was selected. For the defined aim, the lipid film hydration method seemed to be suitable. The steps of this method are presented by the authors on a process map in Fig. 2. The map shows the steps and the procedures of this liposome preparation route, involving the methods, the materials, and the process parameters. In this study, the construction of the process map formed part of knowledge space development and helped in the selection of the CMAs and in the identification of the CPPs. The material and process parameters which were found as critical are listed later in Fig. 3. Moreover, this process map also indicates the step of stabilization after the production of the liposomal product. Liposomes are usually stable for a short period of time. To eliminate this problem, the final step was lyophilisation in order to obtain a stable, solid product.

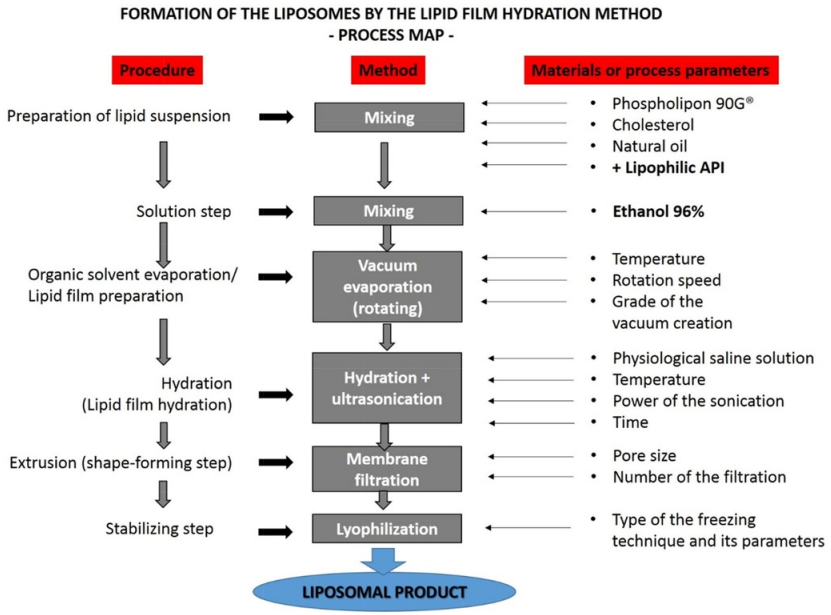


Fig. 2 Process Map of the lipid film hydration method for the preparation of liposomes

QTPP parameter	Impact	CQA	CMAs and CPPs	Occurrence
Therapeutic effect	High	Wall-forming agent 1 (Natural oil/ Cholesterol)	Organic solvent type	Low
Patient group	Low	Wall-forming agent 2 (Phospholipon 90G®)	Temperature of vacuum evaporation	High
Route of administration	High	Hydration media	Grade of vacuum creation	High
Dosage form	Medium	API	Hydration step: feeding rate	High
Volume of one dose	Medium	Surface charge	Temperature rising during the ultrasound	High
Dissolution profile/absorption time	High	Vesicle/particle size (and size distribution)	Filtration number in shape-forming phase	Medium
Stability (product stability)	High	Stability of the vesicle size		
pH of the product	High			

1. Risk Identification

2. Risk Analysis
- using the Risk Estimation Matrix (REM) and the Uncertainty Estimation

CQA	QTPP							
	Therapeutic effect (H)	Patient group (L)	Route of administration (nasal) (H)	Dosage form (M)	Volume of one dose (M)	Dissolution profile/ absorption time (H)	Stability (H)	pH (H)
Wall-forming agent 1 (Natural oil/ Cholesterol)	Low	Low	Medium	Low	Low	High	High	Low
Wall-forming agent 2 (Phospholipon 90G®)	Medium	Low	Medium	Low	Low	High	High	Low
Hydration media	Low	Low	Medium	Low	Low	Medium	Low	High
API	Low	Low	High	Low	Medium	Medium	Low	Medium
Surface charge	Low	Low	Medium	Low	Low	High	Medium	Low
Vesicle/Particle size (and size distribution)	Medium	Low	High	Low	Medium	High	High	Low
Stability of the vesicle size	Low	Low	Medium	Low	Low	High	High	Low

CQA	CMAs/CPPs					
	Organic solvent type (CMA) (23%)	Temperature of the vacuum evaporation (16%)	Grade of vacuum creation (7%)	Hydration step: feeding rate (12%)	Temperature rising during the ultrasound treatment (26%)	Filtration number in the shape-forming phase (16%)
Wall-forming agent 1 (Natural oil/ Cholesterol)	High	High	Low	Low	High	Medium
Wall-forming agent 2 (Phospholipon 90G®)	High	High	Low	Low	High	Medium
Hydration media	Low	Low	Low	Low	Medium	Low
API	High	Low	Low	Low	Low	Low
Surface charge	Low	Low	Low	Low	Low	Low
Vesicle/Particle size (and size distribution)	Low	Low	Medium	High	High	High
Stability of the vesicle size	Low	Low	Low	Low	Medium	Medium
UNCERTAINTY of CMAs/CPPs → →	Low	High	High	High	Medium	Medium

Fig. 3 The selected QTPPs, CQAs, CMAs, and CPPs, and the interdependence rating results of these critical parameters as part of the Risk Assessment (High = the interdependence and the impact of the interaction is high between the examined factors, Medium = the interdependence has a medium effect, Low = the interdependence is low between the factors)

3.2 Initial Risk Assessment

In the RA-based formulation development, the critical factors were analysed, the interrelations between them were estimated, and finally, the factors were ranked by their critical effect on the final product. The process of RA and the stages are presented in Fig. 3. After the determination of the QTPPs and the selection of the CQAs, CMAs, and CPPs, the impact (High, Medium or Low) of the QTPPs was estimated. That was the risk identification phase. As the following step of the risk analysis, a Risk Assessment matrix was created according to the estimated interdependence between the QTPPs and CQAs, and between the CQAs and CMAs/CPPs, and their impact. Each value had to be examined based on the interdependences between them and was rated as to whether the impact of their interdependence is “high”, “medium” or “low”. The estimation of this impact was based on the knowledge of the research group members, their experience gained from experimental practice, and scientific knowledge in the literature. The uncertainty of the critical factors was also estimated (Fig. 3).

As a result of the above-presented procedure, the QbD software calculated the risk priority number (RPN) of each factor and ranked them according to their critical effect on the liposomal product. The RPNs and their rankings are presented on the Pareto charts in Figures 4 and 5. Fig. 4 presents the ranking of the CQAs, while Fig. 5 shows the ranking of the critical factors related to the liposome production process (CMAs/CPPs).

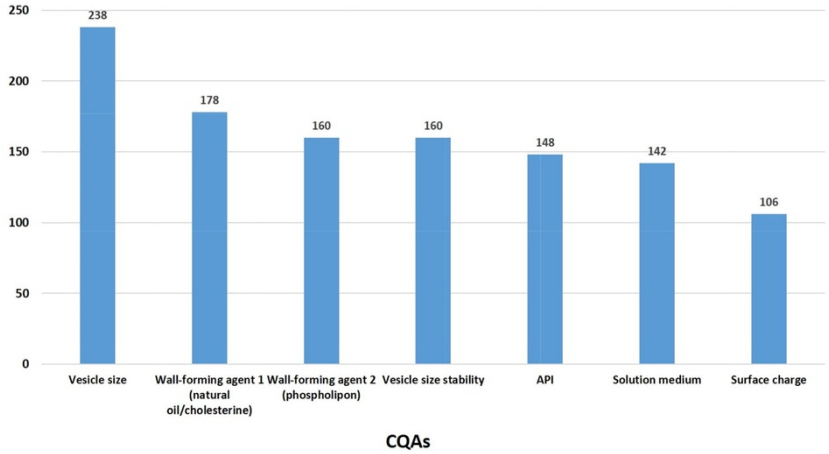


Fig. 4 Pareto chart about the ranking of the CQAs based on their potential critical effect on the aimed liposomal product

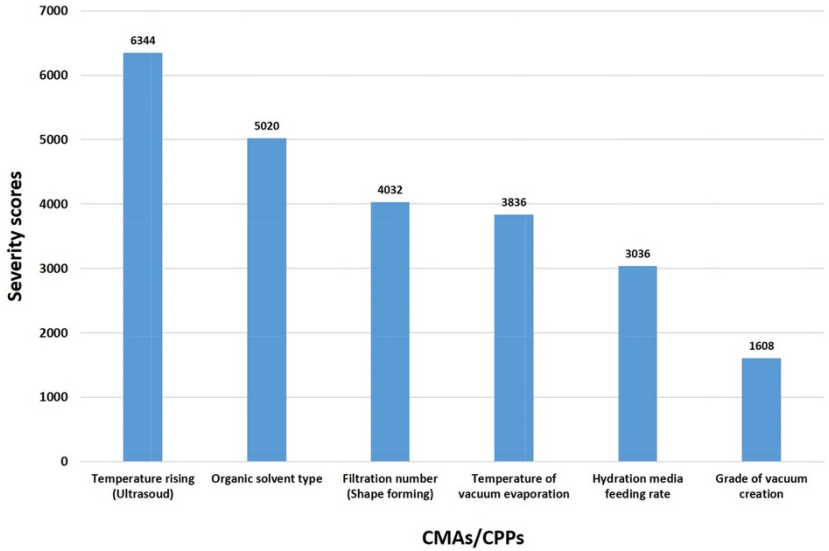


Fig. 5 Pareto chart about the ranking of the CPPs based on their potential critical effect on the aimed liposomal product

Based on the result of this initial RA, the factors for the factorial design of the experiments could be selected.

3.3 Design of Experiments, factorial design

The RA results helped to set up the experimental design. The Main Effect Screening Design 2 programme was chosen for screening the effects of the formulation parameters on the quality of the final liposomal product. The variables were the following: the concentration of the phospholipids (Phospholipon 90G®), which was the third data on the list of the CQAs and was chosen as variable X1. According to our selection methodology, the size of the liposome vesicles and size distribution, which factor was in the first position on the critical list of the CQAs, were selected as the response variable and as the second factor, while the amount of the API was kept constant. From the CPPs, the temperature of ultrasonication was chosen as variable X2, and because the type of the organic solvent was also constant, the following critical factor, the number of the second filtration was designated as variable X3. The pattern of sample preparation and the variables with the selected lower (-) and upper (+) limits are shown in Fig. 6.

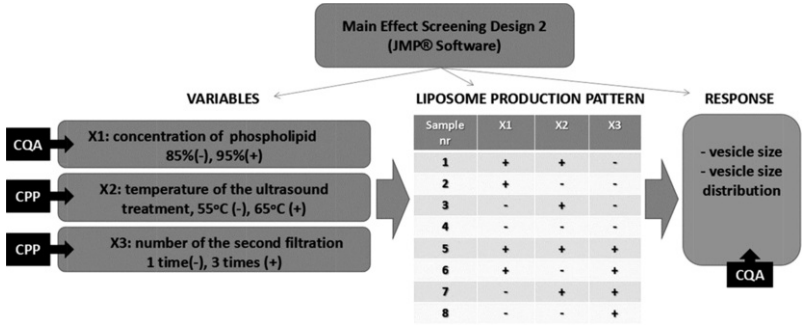


Fig. 6 Design of the experiments: factorial design of liposome preparation

3.4 Characterization results of the liposomal products

The samples, made by the above-mentioned method, were investigated with the two particle size determination analytical method. The results are shown in Table 4.

Table 4 Data of the size determination of the vesicles by the laser diffraction and the dynamic light scattering methods.

Samples	d(0.1) (nm)	d(0.5) (nm)	d(0.9) (nm)	Span	Uniformity	Specific surface area (m^2/g)	Surface weighted mean D[3,2] (nm)	Volume weighted mean D[4,2] (nm)	d (Peak 1) (nm)	PdI
E-95-1-55	77	***184***	587	2.776	0.828	40.3	149	266	314.0	0.435
E-95-3-55	76	***143***	242	2.516	0.760	41.9	143	242	203.4	0.416
A-95-1-55	72	***157***	455	2.437	0.747	45.5	132	219	172.1	0.441
A-95-3-55	74	***139***	517	2.665	2.580	43.2	139	535	165.9	0.698
E-85-1-55	84	***217***	648	2.602	0.793	35.7	168	301	188.4	0.815
E-85-3-55	84	***214***	607	2.455	0.746	36.1	166	287	176.1	0.614
A-85-1-55	72	***156***	214	2.322	0.725	45.9	131	214	190.3	0.389
A-85-3-55	72	***156***	422	2.255	0.704	46.0	130	210	176.3	0.429
E-95-1-65	80	***200***	625	2.728	0.824	37.9	158	285	183.6	0.702
E-95-3-65	79	***193***	563	2.510	0.761	38.9	154	265	221.1	0.438
A-95-1-65	79	***154***	279	2.801	0.840	38.9	154	279	174.7	0.491
A-95-3-65	78	***188***	563	2.584	0.779	39.7	151	262	175.4	0.673
E-85-1-65	88	***180***	326	2.570	0.791	33.4	180	326	242.0	0.879
E-85-3-65	85	***215***	600	2.399	0.735	35.8	168	287	188.3	0.471
A-85-1-65	72	***131***	212	2.291	0.712	45.9	131	212	157.2	0.371
A-85-3-65	73	***160***	441	2.300	0.707	45.0	133	216	210.6	0.525

The integration of the lipophilic API into the liposomal wall decreases the size of the vesicles (presented by d-values in Table 4 and Fig. 7B). Lower diameter values were measured in the case of the API-containing liposomes than in the empty ones. Based on the variable vesicle size values, it can be concluded that the modification of the preparation temperature has no notable effect on particle size distribution. During the investigations, it was observed that the reduction of the phospholipid or the elevation of the cholesterol and the natural oil component ratio decreases the heterodispersity of the systems. The span value describes the width of the particle size ranges. It indicates how far the 10 percent and the 90 percent points are apart and normalised with the midpoint, so the smaller the span value the narrower the particle size distribution range. Using the 85% phospholipid-containing formulations instead of

the 95% ones resulted in systems with better monodispersity values (lower span and PdI values). Uniformity gives information about the absolute deviation from the median size. The monodispersity value of the system increases by the elevation of the number of the filtrations. In these experiments, this connection was stronger among the empty liposomes. D[3,2] and D[4,2] values indicates the central point around which the (surface area or volume/mass) distribution would rotate. The SSA values have been measured also for the particles. Nanospheres have high SSA. SSA data also show that the incorporation of the lipophilic API into the liposomes decreased the size of the vesicles due to the higher SSA values (averages for the empty vesicles are respectively 38.5 and 36.5 m (Ich, 2009)/g, and for the API-containing ones are 45.15 and 42.36 m^2/g for samples produced at 55 °C and 65 °C). Data from dynamic light scattering measurements show the typical size of the vesicles in an intensity-based size distribution (d (Peak 1) nm) and the polydispersity index (PdI) which indicates slightly heterodispers (systems) (Table 4). Zeta potentials show that the originally negative charged empty liposomes turn into positive charged vesicles by the c (Table 5).

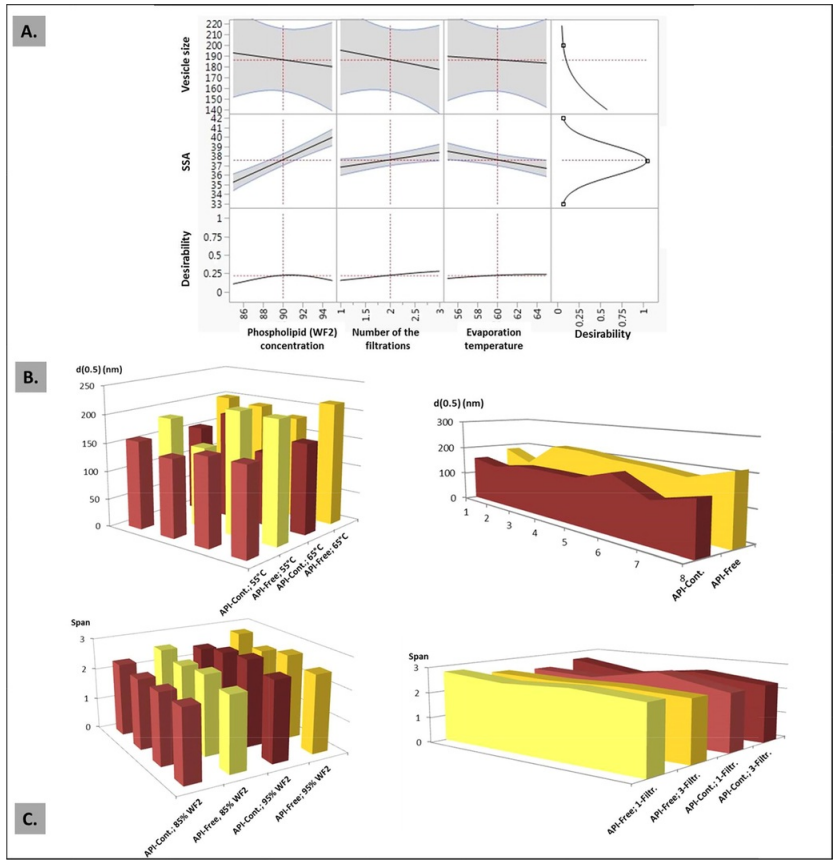


Fig. 7 Scatterplot matrix of the investigation results of the prepared liposomes (vesicle size related to the phospholipid concentration, number of the filtrations and the evaporation temperature) (A), the effect of the temperature and the API on the vesicle size of the liposomes (B), and the impact of the Phospholipon 90G® (WF2) concentration and the number of the filtrations on the heterodispersity of the liposomes(C)

Table 5 Size detection and zeta potential data of the selected vesicles.

Samples	Span	d(0.5) (nm)	Zeta potential
E-95-1-65	2.728	**200 ± 2**	−2.14 ± 0.39
E-95-3-65	2.510	**193 ± 2**	−2.52 ± 0.27
A-95-1-65	2.801	**154 ± 3**	2.81 ± 0.31

A-95-3-65	2.584	**188 ± 3**	2.63 ± 0.11
E-85-1-65	2.570	**180 ± 12**	−3.82 ± 0.35
E-85-3-65	2.399	**215 ± 16**	−3.90 ± 0.39
A-85-1-65	2.291	**131 ± 13**	2.13 ± 0.7
A-85-3-65	2.300	**160 ± 18**	1.44 ± 0.11

E = 'empty liposomes', A = 'API-containing liposomes', 85 = '85 w/w% phospholipid concentration', 95 = '95 w/w% phospholipid concentration', 1 = 'one-time filtration', 3 = 'three-time filtration' were made with a 0.22-µm membrane filter, 55 = '55 °C as the preparation temperature', 65 = '65 °C as the preparation temperature', d (Peak 1) and PdI were measured by the dynamic light scattering technique, while the other parameters were measured by the laser diffraction method.

The produced liposomes and the measurement results were further evaluated to understand better the interrelations between the production process parameters and the product performance. A software-supported data evaluation was carried out, which resulted in a scatterplot matrix (Fig. 7A). Fig. 7-A specifically presents the alteration of the liposomal vesicle size values related to the phospholipid concentration, the number of the filtrations and the temperature of the evaporation.

The increase of the phospholipid concentration (which was named as wall-forming agent 2 –WF2 – during the design phase) resulted in decreased size and increased SSA values. On the other hand, the higher number of the filtrations led to liposomes with a lower size but caused a moderate increase in the SSA (the slope of the line is lower). Lastly, part A of Fig. 7 (Fig. 7A) shows that the increase of the evaporation temperature during the production process has only a little impact on vesicle size and the SSA. Section B in the same figure (Fig. 7B) presents the effect of temperature and the presence of the API on the vesicle size (d(0.5) nm) of the liposomes. The graphics demonstrate that the presence of the API, built in the structure of the liposomes, resulted in smaller vesicles, so the lipophilic API-containing vesicles are smaller than the API-free ones. The same result was observable with both production temperature values (55 and 65°C).

Part C of Fig. 7 (Fig. 7C) presents the same results as Table 4, namely, the usage of a higher amount of WF2 slightly decreased the vesicle size of the liposomes and increased the heterodispersity of the system. The monodispersity values of the produced liposomal systems increased by the elevation of the number of the filtrations and with the usage of less WF2 in the compositions.

3.5 Results of the SEM investigation

After the lyophilisation step of liposome preparation, a SEM investigation was performed. The images taken about the freeze-dried samples proved the results obtained from the particle size characterization. The average size of the liposomes was within the range of 200 nm. Furthermore, the spherical shape of the separated liposomes is visible in Fig. 8.

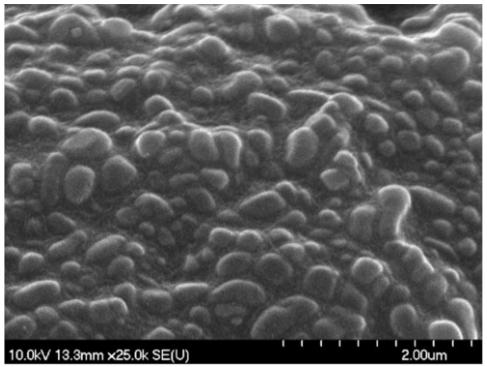


Fig. 8 SEM picture about the produced and freeze dried (lyophilised) liposomal product sample

3.6 Results of the drug entrapment efficiency

The absorbance value based on the spectrophotometric investigation of the selected liposomal product (A-85-3-65, Table 4) was 0.34 ± 0.01. According to this, the calculated drug entrapment of the sample was 98.27±0.26%.

4 Conclusion

In this study, the QbD-based and mostly RA-focused approach was successfully adapted in the early development phase of a lipophilic API-containing liposomal formulation. An RA-based theoretical design and development were created before the practical phase of the liposomal formulation started in practice. The liposomes were designed for nasal application, and the expectation related to the model API, which was a lipophilic BCS Class II drug, was to be able to reach theoretically the brain tissue (in the further investigations) via the nasal administration route. To achieve these long-range goals this first step formulation design was performed, the elements of the QTPP were defined, and the lipid film hydration method was selected to prepare the aimed liposomes. The CPPs/CMAs related to the process, as well as the CQAs, were determined and the RA was fulfilled. Based on the initial RA results, in practice, the preparation of the liposomes was carried out following a factorial design plan. The factors of the design plan were derived from the most critical elements of the CQAs and CPPs and formed the pattern of the experimental design, thus liposome preparation was focused only on the most highly critical parameters. The following of the theoretical screening and selection method of the critical factors led to a lower number of investigations, but an even higher rate of successful sample preparation was achieved. The investigational results of the prepared liposomes (API-free and API-containing samples were prepared following the same factorial design pattern), namely vesicle size, size distribution, specific surface area, and surface characteristics, verified the exactness of the RA and the critical factor-based theoretical prediction, and showed clear relations between the product-design (composition of the liposomes, temperature, and process parameters, such as temperature, or the number of filtrations, etc.) and the product characteristics of the prepared liposomes. The results proved that the QbD approach can improve the formulation process in the development of liposomes, lead to an effective product preparation process, and help in the optimization and the rationalization of liposomal developments even in those special cases when a lipophilic active ingredient is incorporated into the liposomes.

Uncited reference

Aramaki et al. (1994).

Acknowledgements

This project was supported by the Gedeon Richter Ltd. – GINOP project (2.2.1-15-2016-00007), the authors hereby express their sincerest gratitude and thanks for the sponsorship.

Moreover, the authors would like to express their special thanks to dr. Rita Ambrus Ph.D. for the preparation of the SEM pictures of the liposome products.

References

Agarwal N.B., Jain S., Nagpal D., Agarwal N.K., Mediratta P.K. and Sharma K.K., Liposomal formulation of curcumin attenuates seizures in different experimental models of epilepsy in mice, *Fundam. Clin. Pharmacol.* **27** (2), 2013, 169–172, https://doi.org/10.1111/j.1472-8206.2011.01002.x.

Akbarzadeh A., Rezaei-Sadabady R., Davaran S., et al., Liposome: classification, preparation, and applications, *Nanoscale Res Lett.* **8** (1), 2013, 1–8, https://doi.org/10.1186/1556-276X-8-102.

Allen T.M., Hansen C.B. and Guo L.S.S., Subcutaneous administration of liposomes: a comparison with the intravenous and intraperitoneal routes of injection, *BBA – Biomembr.* **1150** (1), 1993, 9–16, https://doi.org/10.1016/0005-2736(93)90115-G.

Aramaki Y., Fujii Y., Yachi K., Kikuchi H. and Tsuchiya S., Activation of systemic and mucosal immune response following nasal administration of liposomes, *Vaccine* **12** (13), 1994, 1241–1245 http://www.ncbi.nlm.nih.gov/pubmed/7839731.

Arora P., Sharma S. and Garg S., Permeability issues in nasal drug delivery, *Drug Discov. Today* **7** (18), 2002, 967–975, https://doi.org/10.1016/S1359-6446(02)02452-2.

Bacher A., Green chemistry, *Encycl. Toxicol.* 2014, 1–12, https://doi.org/10.1016/b0-12-369400-0/00463-4.

Bartos C., Ambrus R., Sipos P., et al., Study of sodium hyaluronate-based intranasal formulations containing micro- or nanosized meloxicam particles, *Int. J. Pharm.* **491** (1–2), 2015, 198–207, https://doi.org/10.1016/j.ijpharm.2015.06.046.

Bartos C., Ambrus R., Kovács A., et al., Investigation of absorption routes of meloxicam and its salt form from intranasal delivery systems, *Molecules* **23** (4), 2018, 1–13, https://doi.org/10.3390/molecules23040784.

Bozzuto G. and Molinari A., Liposomes as nanomedical devices, *Int. J. Nanomed.* **10**, 2015, 975–999, https://doi.org/10.2147/IJN.S68861.

Briuglia M.L., Rotella C., McFarlane A. and Lamprou D.A., Influence of cholesterol on liposome stability and on in vitro drug release, *Drug Deliv. Transl. Res.* **5** (3), 2015, 231–242, https://doi.org/10.1007/s13346-015-0220-8.

Csóka I., Pallagi E. and Paál T.L., Extension of quality-by-design concept to the early development phase of pharmaceutical R&D processes, *Drug Discov. Today.* 2018, https://doi.org/10.1016/j.drudis.2018.03.012.

Deamer D.W., From “banghasomes” to liposomes: a memoir of Alec Bangham, 1921–2010, *FASEB J.* **24** (5), 2010, 1308–1310, https://doi.org/10.1096/fj.10-0503.

Dua J.S., Rana A.C. and Bhandari A.K., Review article liposome : methods of preparation and applications, *Int. J. Pharm. Stud. Res.* **3** (II), 2012, 14–20, https://doi.org/10.1017/CBO9781107415324.004.

Gieszinger P., Csóka I., Pallagi E., et al., Preliminary study of nanonized lamotrigine containing products for nasal powder formulation, *Drug Des. Dev. Ther.* **11**, 2017, https://doi.org/10.2147/DDDT.S138559.

Tamer A. Green Solvents, 2018. https://www.acs.org/content/dam/acsorg/greenchemistry/education/summerschool/Tamer Andrea_Greener Solvents.pdf.

Hussain A.A., Intranasal drug delivery, *Adv. Drug Deliv. Rev.* **29** (1–2), 1998, 39–49, https://doi.org/10.1016/S0169-409X(97)00060-4.

Ich. ICH Q10 Pharmaceutical Quality Systems. EPT-The Electron Newsl Pharm Tech Jun. 2009; May: 21. doi: 10.1007/978-3-319-15814-3.

Ich. Pharmaceutical Development Q8. ICH Harmon Tripart Guidel. 2009, 8 (August), 1–28.

Illum L., Nasal drug delivery – Possibilities, problems and solutions, *J. Control. Rel.* **87**, 2003:, 187–198, https://doi.org/10.1016/S0168-3659(02)00363-2.

Jójárt-Laczkovich O., Bónis E., Németh Z. and Szabó-Révész P., Influence of wheat germ oil content on mean vesicle size of liposomes, *Acta Pharm. Hung.* **88**, 2018, 1–8.

Jouyban A., Soltanpour S. and Jr WEA, Improved prediction of drug solubilities in ethanol + water mixtures at various, *Temperatures* **2010**, 2010, 19–24.

Karimi K., Pallagi E., Szabó-Révész P., Csóka I. and Ambrus R., Development of a microparticle-based dry powder inhalation formulation of ciprofloxacin hydrochloride applying the quality by design approach, *Drug Des. Dev Ther.* **10**, 2016, https://doi.org/10.2147/DDDT.S116443.

Khale A., Bajaj A., Instititute M.E.S., Pharmacy H.K.C.O., Complex A.M. and Jogeshwari W., Lipid characterization study in preparation of liposomes of salbutamol sulphate, *J. Pharm. Res.* **4** (4), 2011, 1267–1269.

Laouini A., Jaafar-Maalej C., Limayem-Blouza I., Sfar S., Charcosset C. and Fessi H., Preparation, characterization and applications of liposomes: state of the art, *J Colloid Sci. Biotechnol.* **1** (2), 2012, 147–168, https://doi.org/10.1166/jcsb.2012.1020.

Maherani B., Arab-Tehrany E., Mozafari R., Gaiani M., Linder C. and Liposomes M., A review of manufacturing techniques and targeting strategies, *Curr. Nanosci.* **7** (3), 2011, 436–452, https://doi.org/10.2174/157341311795542453.

Nunes V.D., Sawyer L., Neilson J., Sarri G. and Cross J.H., Diagnosis and management of the epilepsies in adults and children: summary of updated NICE guidance, *BMJ* **344** (7842), 2012, https://doi.org/10.1136/bmj.e281.

Pallagi E., Ambrus R. and Szabó-Révész P., Csóka I. Adaptation of the quality by design concept in early pharmaceutical development of an intranasal nanosized formulation, *Int. J. Pharm.* **491** (1–2), 2015, https://doi.org/10.1016/j.ijpharm.2015.06.018.

Pallagi E., Karimi K., Ambrus R., Szabó-Révész P. and Csóka I., New aspects of developing a dry powder inhalation formulation applying the quality-by-design approach, *Int. J. Pharm.* **511** (1), 2016, https://doi.org/10.1016/j.ijpharm.2016.07.003.

Parvathi M., Intranasal drug delivery to brain: an overview, *Ijrpc* **2** (3), 2012, 889–895 http://ijrpc.com/files/42-2161.pdf.

Patil A.S. and Pethe A.M., Quality by design (QbD): a new concept for development of quality pharmaceuticals, *Int. J. Pharm. Qual. Assur.* **4** (2), 2013, 13–19, https://doi.org/10.1007/s11095-007-9511-1.

Pires P.C. and Santos A.O., Nanosystems in nose-to-brain drug delivery: a review of non-clinical brain targeting studies, *J. Control Rel.* **270**, 2018, 89–100, https://doi.org/10.1016/j.jconrel.2017.11.047.

Powell T. and Sammut-Bonnic T., Pareto Analysis, In: Cooper C.L., (Ed), *Wiley Encyclopedia of Management,* 2014, John Wiley & Sons, Ltd, doi: 10.1002/9781118785317.weom120202.

I.C.H. Quality Risk Management Q9. ICH Harmon Tripart Guidel. 2005 1–23. doi: 10.1007/s11095-007-9511-1.

Rawle A. Basic principles of particle size analysis. Malvern Instiruments, Tech Pap. 44(0).

Rodriguez-aller M., Guillarme D., Veuthey J. and Gurny R., Journal of drug delivery science and technology strategies for formulating and delivering poorly water-soluble drugs, *J. Drug Deliv. Sci. Technol.* **30**, 2015, 342–351, https://doi.org/10.1016/j.jddst.2015.05.009.

Schlumberger E., Chavez F., Palacios L., Rey E., Pajot N. and Dulac O., Lamotrigine in treatment of 120 children with epilepsy, *Epilepsia* **35** (2), 1994, 359–367, https://doi.org/10.1111/j.1528-1157.1994.tb02445.x.

Serralheiro A., Alves G., Fortuna A. and Falcão A., Direct nose-to-brain delivery of lamotrigine following intranasal administration to mice, *Int. J. Pharm.* **490** (1–2), 2015, 39–46, https://doi.org/10.1016/j.ijpharm.2015.05.021

Sonvico F., Clementino A., Buttini F., Surface-Modified, et al., Nanocarriers for nose-to-brain delivery: from bioadhesion to targeting, *Pharm* **2018** (34), 2018, 1–34, https://doi.org/10.3390/pharmaceutics10010034.

Tague N.R., Fishbone diagram (Ishikawa) – cause & effect Diagram, *Qual Toolbox* 2005, 247–249.

Torchilin V.P., Targeted pharmaceutical nanocarriers for cancer therapy and imaging, *AAPS J.* **9** (2), 2007, E128–E147, https://doi.org/10.1208/aapsj0902015.

Tsume Yasuhiro, Mudie Deanna M., Langguth Peter, Amidon Greg E.G.L. and NIH Public Access, *Eur. J. Pharm. Sci.* **57**, 2014, 152–163, https://doi.org/10.1016/j.ejps.2014.01.009.

Tubic-grozdanis M., Bolger M.B. and Langguth P., Application of gastrointestinal simulation for extensions for biowaivers of highly permeable compounds, *AAPS J.* **10** (1), 2008, 213–226, https://doi.org/10.1208/s12248-008-9023-x.

Türker S., Onur E. and Özer Y., Nasal route and drug delivery systems, *Pharm. World Sci.* **26** (3), 2004, 137–142, https://doi.org/10.1023/B:PHAR.0000026823.82950.ff.

Van Winden E.C.A., Zhang W. and Crommelin D.J.A., Effect of freezing rate on the stability of liposomes during freeze-drying and rehydration, *Pharm Res.* **14** (9), 1997, 1151–1160, https://doi.org/10.1023/A:1012142520912.

Winterhalter M. and Lasic D.D., Liposome stability and formation: experimental parameters and theories on the size distribution, *Chem. Phys. Lipids* **64** (1–3), 1993, 35–43, https://doi.org/10.1016/0009-3084(93)90056-9.

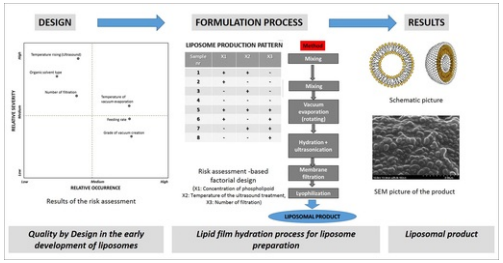
Yu L.X., Pharmaceutical quality by design: product and process development, understanding, and control, *Pharm Res.* **25** (4), 2008, 781–791, https://doi.org/10.1007/s11095-007-9511-1.

Yu L.X., Amidon G., Khan M.A., et al., Understanding pharmaceutical quality by design, *AAPS J.* **16** (4), 2014, 771–783, https://doi.org/10.1208/s12248-014-9598-3.

Zawada Z., A single-step method of liposome preparation, *Cell Mol. Biol. Lett.* **9** (4A), 2004, 603–615 http://www.ncbi.nlm.nih.gov/pubmed/15647784.

Zylberberg C, Matosevic S, Zylberberg C, Matosevic S. Pharmaceutical liposomal drug delivery : a review of new delivery systems and a look at the regulatory landscape Pharmaceutical liposomal drug delivery : a review of new delivery systems and a look at the regulatory landscape. 2016; 7544. doi: 10.1080/10717544.2016.11771366.

Graphical abstract



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