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Faculty of Pharmacy

Institute of Pharmaceutical Technology and Regulatory Affairs

Summary of PhD thesis

RESEARCH AND DEVELOPMENT OF NANOSIZED DRUG CONTAINING NASAL DRUG DELIVERY SYSTEMS TO REACH SYSTEMIC AND CENTRAL NERVE SYSTEM EFFECT

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1. INTRODUCTION

Nasal drug delivery has received remarkable attention in the past few decades because the nose offers a great alternative administration route due to its special anatomy and physiological properties. Via the nasal application, local, systemic and central nervous system effects are also available and even though the majority of products are for local use (e.g. decongestants), an increasing number of products with systemic and CNS effect could be found on the market. The fact that nasal formulations are not just for local therapy, makes them attractive in the therapy of CNS diseases (e.g. epilepsy, Parkinson's disease, Alzheimer's disease). Nasal administration is pain-free, whilst a rapid onset of action can be achieved; therefore, enhanced patient adherence can be accomplished.

Most of the APIs used nowadays are in BCS II, which means that these drugs have low solubility and high permeability. One of the most popular ways to improve the solubility and dissolution rate of APIs is particle size reduction, including nanonization. Nanosized drugs can provide higher bioavailability due to their smaller particle size and larger specific surface. There are different kinds of technologies with which nanosystems can be produced. The two main groups are bottom-up and top-down technologies. In the case of the bottom-up technology, micro or nanoparticles are built up from dissolved drug molecules, while in the case of the top-down approach, the raw material is subsequently broken down. Both approaches were applied in this project as NP formulations were produced with a top-down (co-milling) method, while NCs were produced with a bottom-up (solvent displacement) technique.

QbD is a holistic and systematic quality management method, where the development design is risk and knowledge-based. The foundation of a QbD-guided development is RA which can be initial, updated, or final. Good RA results are essential for designing the researches more efficiently and economically in practice, which makes the studies ecologically friendly, time and cost-effective. Time and cost-effectiveness are key elements during the pharmaceutical research and development of different formulations. Thus, the development of proper *in vitro* models is exceedingly significant from an economical and ecological aspect. In the case of nasal formulations, due to the unique anatomical and physiological properties, many aspects need to be considered during the development procedure. One of the most important factors is the permeability rate of the API from the product across the membrane, so its accurate detection is particularly important during the development.

The thesis reports the development and investigation of two types of nasal formulations that contain nanosized lamotrigine to use them in the therapy of epilepsy as alternative dosage forms of the traditionally applied tablets.

Abbreviations: API- Active pharmaceutical ingredient, AUC- Area under curve, BBB- Blood-brain-barrier, BCS- Biopharmaceutical classification system, CNS- Central Nervous System, CPP- Critical Process Parameter, CQA- Critical Quality Attribute, DoE- Design of experiment, DS- Design space, DTE- Drug targeting efficiency- EMA- European Medicines Agency, FDA- Food and Drug Administration- FDNCs- Freeze-dried nanocapsules, GI- Gastrointestinal tract, IV- Intravenous, LAM- Lamotrigine, LC-MS- Liquid chromatography-mass spectroscopy, MCC- Mucociliary clearance, NaHA- Sodium hyaluronate, NCs- Nanocapsules, NP- Nasal powder, PBS- phosphate-buffered saline, PDI- Polydispersity index, PgP- P-glycoprotein, PM- Physical Mixture, PS- Particle size, PVA- Polyvinyl alcohol, PVP- Polyvinyl pyrrolidone, QbD- Quality by Design, QTPP- Quality Target Product Profile, RA- Risk assessment, SEM- Scanning Electron Microscopy, SNES- Simulated nasal electrolyte solution

2. AIMS OF THE WORK

This Ph.D. work aimed to research, develop and investigate LAM containing nasal formulations to induce systemic and CNS effect. Accordingly, the goal was to develop nanosized LAM containing nasal dosage forms, which could be great alternatives to marketed tablets in the therapy of epilepsy. The parts of the project were the following:

- ✓ Literature review of nasal drug delivery, nanosystems, and QbD methodology to lay the theoretical foundations for the development of QbD based, nanosized LAM containing nasal formulations, which would be produced with a top-down and a bottom-up method. The reason this field was chosen that despite the well-known advantages of the nasally used formulations, just a few nasal powders or nasally administered NCs can be found on the market.
- ✓ QbD based RA for NP delivery, identification, prioritization and selection of the most influencing CQAs, CPPs of NP production with the tools of QbD. Preliminary study of the nanosized API containing NP and choose the most promising additive. Optimization of the dry milling process with DoE, which means the Design Space determination and validation of the powder production.
- ✓ Micrometric and structural investigation of NP formulation, moreover the implementation of *in vitro* and *in vivo* investigations, and parallelly the development, adaptation, and validation of a modified *in vitro* horizontal permeability method.
- ✓ QbD based RA for LAM encapsulation, which consisted of the identification, prioritization, and selection of the most influencing factors using an Ishikawa diagram. Development, optimization, and production of LAM containing NCs, which meant the determination of formulation components, their ratios and the process parameters.

- ✓ Micrometric and structural investigations of NC formulation, moreover the implementation of *in vitro* and *in vivo* investigations.
- ✓ Comparison of NC and NP samples, and based on the *in vivo* performance of formulations and finally, make a suggestion which formulation could be used as an innovative delivery system in the therapy of epilepsy.

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Active pharmaceutical ingredient

LAM, poorly water-soluble (0.17 mg/mL at 25 °C) second-generation antiepileptic drug of the phenyltriazine class, was purchased from Teva Ltd. (Budapest, Hungary).

3.1.2. Excipients

The NP additives, PVP (M_w=24000) and PVA (M_w=27,000), water-soluble synthetic polymer were supplied by ISP Customer Service GmBH (Cologne, Germany). Sodium hyaluronate (NaHA) polymer was purchased from Gedeon Richter Ltd. (Budapest, Hungary)

The NC additives: Glyceryl monooleate (Type 40) (Peceol®) and Diethylene glycol monoethyl ether (Transcutol HP®) were a kind gift from Gattefossé (St. Preist, France). Polyoxyethylene (40) monostearate (PEG-stearate 40) was purchased from Croda (East Yorkshire, United Kingdom). Chitosan hydrochloride salt was obtained from HMC+ (Halle, Germany). Mannitol was obtained from Sigma-Aldrich (New York, USA).

3.2. Initial RA for NP products

As part of the QbD methodology, an Ishikawa diagram was set up to identify a knowledge space of the NP and NC formulations. With the Ishikawa diagram, the identification and systematization of influencing factors were carried out. The factors with the highest influence were chosen and varied. To perform the initial RA, the first step was the determination of the QTPP of the aimed product. After that, the CQAs and the CPPs of the selected production method were identified.

3.3. Dry milling method for NP production

PVP, PVA, and NaHA were used for sample preparation as additives to maintain the stability and individuality of nanosized LAM particles. Given amount of LAM and additives (1:1, 1:2 and 2:1 ratios) were placed in a Turbula mixer (Turbula System Schatz; Willy A. Bachofen AG Maschinenfabrik, Basel, Switzerland) using 60 rpm for 10 minutes; thus, interactive PMs were prepared. Then, the PMs were put into a planetary ball mill (Retsch PM 100, Retsch, Germany) and milled on 400 rpm speed for 2 hours.

During the optimization process, in the case of nanoLAMpowder, the LAM:PVA ratio was 1:0.8 and the sample was milled for 1.5 h on 400 rpm.

3.4. NC production

Solvent displacement method for NC production

The NCs were prepared by a solvent displacement method, whose composition (Table 1.) was determined after preliminary experiments. The organic phase was poured over 2 mL of ultrapure water upon continuous magnetic stirring. After 10 minutes, 2 mL of chitosan solution (1 mg/mL) was added upon magnetic stirring, leading to the spontaneous formation of the NCs.

Table 1. The compositition of the NC formulation

LAM solution(100 mg/mL) (µL)	100	+ 2 mL MilliQ water	+ 2 mL 1mg/mL Chitosan solution after 10 mins
Peceol® (µL)	41.7		
Transcutol®(µL)	41.7		
PEG-stearate 40 solution (5.33 mg/mL EtOH solution)	ad 1 mL		

After 10 additional minutes of stirring, the NCs were isolated and concentrated to a final theoretical chitosan concentration of 1 mg/mL by centrifugation (Hettich Universal 32 R; Tuttlingen, Germany) at 33000xg for 33 min at 15 °C. In parallel, control blank NCs, without LAM were prepared using the same method.

Freeze-drying method for solid-phase nanocapsule production

The freeze-drying was performed in Scanvac CoolSafe 100-9 Pro type equipment (LaboGene ApS, Lynge, Denmark) equipped with a 3 shelf sample holder unit, recessed into the drying chamber. The prepared NCs were lyophilized with 5 w/w% mannitol. The process was controlled by a computer program (Scanlaf CTS16a02), the temperature and pressure values were recorded continuously. Thereafter the FDNCs came up. Figure 1. illustrates the process of the NC preparation.

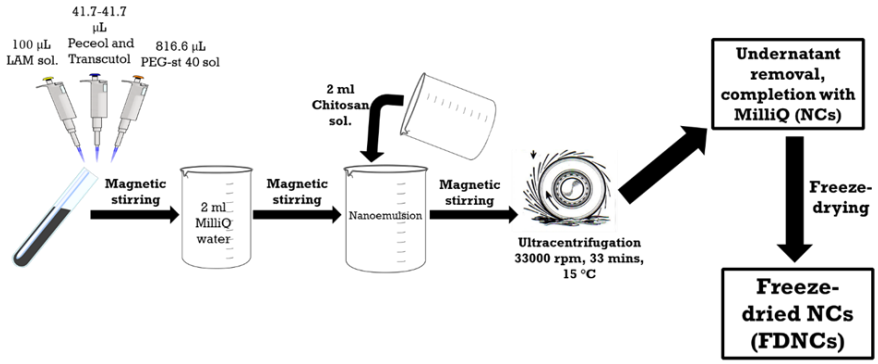

Figure 1. The process of NC and FDNC preparation

3.5. Micrometric investigations

Determination of particle size of NP formulation

The particle size of the microparticles was characterized by using Leica Image Processing and Analysis System device (Leica Q500MC; Leica Microsystems, Wetzlar, Germany).

The particle size of the LAM nanoparticles on the polymer surface was investigated by SEM pictures (Hitachi S4700; Hitachi Ltd., Tokyo, Japan) at 10 kV. The distribution of LAM particle diameter was obtained by analyzing SEM images with the ImageJ software (1.50i; Java 1.6.0_20 [32-bit]; Windows NT) environment using approximately 500 particles.

Particle size, particle size distribution and surface charge characterization of NCs

The particle size and polydispersity index of the NCs were determined by photon correlation spectroscopy (PCS) (Zetasizer NanoZS®, Malvern Instruments; Malvern, United Kingdom). In the case of surface charge, zeta potential (ZP) measurements were done by laser Doppler anemometry (LDA) using the same equipment.

Image analysis (SEM)

The morphology of particles was investigated by SEM (Hitachi S4700; Hitachi Ltd., Tokyo, Japan) at 10 kV. The samples were gold–palladium-coated (90 s) with a sputter coater (Bio-Rad SC502; VG Microtech, Uckfield, UK) using an electric potential of 2.0 kV at 10 mA for 10 min. The air pressure was 1.3–13.0 mPa.

3.6. Structural investigations

Differential scanning calorimetry (DSC)

The thermal response of each product was measured using a differential scanning calorimeter (Mettler Toledo TG 821^e DSC; Mettler Inc., Schwerzenbach, Switzerland). About 3–5 mg of powder was precisely weighed into DSC sample pans, which were hermetically sealed, and the lid was pierced. Each sample was measured in the temperature interval of 25–230 °C at a heating rate of 5 °C/min and under a constant argon flow of 150 mL/min. Data analysis was performed using the STAR^e software (Mettler Toledo; Mettler Inc., Schwerzenbach, Switzerland). The crystallinity index was calculated based on the normalized integral values. PM samples were regarded as 100%.

X-ray powder diffraction (XRPD)

The XRPD measurement was carried out with a BRUKER D8 advance X-ray powder diffractometer (Bruker AXS GmbH, Karlsruhe, Germany) with Cu·K λI radiation (λ=1.5406 Å) and a VÅNTEC-1 detector (Bruker AXS GmbH, Karlsruhe, Germany). The powder samples were loaded in contact with a plane quartz glass sample slide with an etched

square and measured. Samples were scanned at 40 kV and 40 mA. The angular range was 3–40° 2θ, at a step time of 0.1 seconds and a step size of 0.007°. All manipulations, including Kα2 stripping, background removal and smoothing of the area under the peaks of the diffractograms, were performed using the DIFFRACplus EVA software. The crystallinity index (X_c) values were calculated based on the following formula, where A marks the area under the whole curve:

$$X_c = \frac{A_{crystalline}}{A_{crystalline} + A_{amorphous}} * 100$$

PM samples were regarded as 100%.

3.7. *In vitro* studies

In vitro release study of NPs

The modified paddle method (USP dissolution apparatus, type II; Pharma Test, Hainburg, Germany) was used to examine the dissolution rate of the samples and determine their drug release profile. The tests were carried out under nasal conditions for temperature (30°C) and pH (*p*H 5.6). After filtration, the drug content of the aliquots was determined using spectrophotometry (Unicam UV/VIS Spectrophotometer, Cambridge, UK) at 307 nm.

In vitro release study of NCs

The modified paddle method (USP dissolution apparatus, type II; Pharma Test, Hainburg, Germany) was used to examine the dissolution rate of NCs and determine the drug release profile from the samples. To model the nasal pH and temperature conditions, the medium was 9 mL phosphate-buffered saline (PBS) adjusted to *p*H 5.60. The samples were investigated with an RP-HPLC-DAD system. The RP-HPLC-DAD consisted of an Agilent 1200 Series chromatograph and a DAD detector. The stationary phase was a Kinetex® C_{18} Colonna (150 mm x 4,6 mm, particle size: 5 µm, pore diameter size: 100 Å).

Development of a modified *in vitro* permeability investigation method

Horizontal diffusion studies

A horizontal diffusion device (Side-Bi-Side™ Grown Glass, USA) was used for the investigation of NPs and the tests were carried out under nasal conditions. Regenerated cellulose membrane (Whatman™) with 0.45 µm pore diameter was soaked in IPM, and the donor phase was tempered to 30 °C at *p*H 5.6. The acceptor phase was *p*H 7.4, and the content of the diffused drug was measured spectrophotometrically at 307 nm (Unicam UV/Vis Spectrophotometer, Cambridge, UK).

Application of modified permeability investigation method

After the modifications and tests, it was confirmed that the previously used method and parameters were the most suitable for LAM and, therefore, the NCs were measured with the same method like the NPs were, but with AvaLight DH-S-BAL spectrophotometer (AVANTES, Apeldoorn, Netherlands) connected to an AvaSpec-2048L transmission immersion probe (AVANTES, Apeldoorn, Netherlands) with optical fiber to quantify the amount of API. The NPs were re-measured with the inline method and there was no significant difference in the results, so we decided to use the original data. However, it is important to note that the new method is easy-to-implement, more accurate, more reproducible, which made our experiments more simple and more effective.

3.8. *In vivo* studies

The nanoLAMpowder and also the PM contained 0.555 mg, while the NC formulation contained 0.066 mg LAM and the FDNCs formulation contained 0.039 mg LAM. The doses were the maximums that a rat nostril can tolerate and we were able to administer. The formulations were administered into the right nostril of 160–180 g male Sprague–Dawley rats (n=4) with a small spatula or pipette. As a control, IV injections of LAM solutions (IV LAM), which contained 0.555 mg of API were given to rats (n=4). At predetermined time points (3, 6, 10, 20, 40 and 60 mins) after LAM administration, blood samples were collected by cardiac puncture into heparinized tubes under isoflurane anesthesia. Then the animals were sacrificed by decapitation, and brain tissues were quickly removed, rinsed in ice-cold PBS, divided into left and right hemispheres, weighed, and stored at -80 °C until assayed. The experiments were performed according to the EU Directive 2010/63/EU for animal experiments and were approved by the Hungarian Ethical Committee for Animal Research (permission number: IV/1247/2017). The liquid chromatographic separation was performed on an Agilent 1100 Series HPLC system (Agilent; Santa Clara, USA) using a Kinetex C18 (2.6 µm 100A, 50 x 2.1 mm) column (Phenomenex; Torrance, USA). Samples were analyzed with an on-line connected Q Exactive Plus quadrupole-orbitrap hybrid mass spectrometer (Thermo Fisher Scientific; Waltham, USA) equipped with a heated electrospray ion source (HESI). Data acquisition and processing were performed using Xcalibur™ and Quan Browser softwares (Thermo Fisher Scientific; Waltham, USA).

Calculation of drug targeting efficiency

Drug targeting efficiency (DTE) – relative exposure of the brain to the drug following intranasal administration vs. systemic administration – was calculated according to the following formula:

$$DTE = \frac{\left(\frac{AUCbrain}{AUC\ blood}\right)IN}{\left(\frac{AUCbrain}{AUCblood}\right)IV}$$

The value of DTE can range from $-\infty$ to ∞, and the values higher than 1.0 indicate more efficient drug delivery to the brain following intranasal administration as compared to the systemic administration.

Calculations of the area under the time-concentration curve (AUC) and statistical analysis

The calculation of area under the curve (AUC) of the time (min) – concentration (µg/L) curves of each group of animals were performed with the PKSolver add-in from Microsoft Excel (MS Office 2010) using the non-compartmental analysis of data after extravascular input (model #101) of LAM. The AUC values were calculated using the linear trapezoidal method. All reported data are means ± SD.

3.9. Stability measurements

Stability tests of NPs were performed according to International Council for Harmonisation (ICH) Q1A guideline in Binder KBF 240 (Binder GmbH, Tuttlingen, Germany) equipment, with a constant-climate chamber. An electronically controlled APT.lineTM line preheating chamber and refrigerating system ensured temperature accuracy and reproducibility of the results in the temperature range between 10 and 70 °C and the relative humidity (RH) range between 10 and 80%. The stability test was performed at 25 ± 2 °C with 50 ± 5% RH (room conditions). Sampling was carried out after 1 day; 3 and 6 months.

Statistical analysis was performed with TIBCO Statistica® 13.4 (Statsoft Hungary, Budapest, Hungary). All reported data are means ± SD. The Student's t-test was used to determine the statistical significance. Changes were considered statistically significant at $p < 0.05$.

4. RESULTS

4.1. Investigation of NP products

4.1.1. Initial RA for NP products

The QTPP that describes the desired nanosized LAM containing NP was defined, and the CQAs and the CPPs were also identified. As a result, it was found that theoretically among the CQAs, the dissolution rate, the particle size and its distribution can be predicted to have the greatest effect on the quality of the targeted and desired NP product, while in the case of CPPs they were the milling time, the milling speed and the API: additive ratio.

4.1.2. Particle size analysis

The smallest particle size was measured for the powder containing PVP and the largest one for the sample that contained PVA, particle size of which hardly decreased. In Table 2., it can

also be seen that the particle size of LAM was between 120 and 230 nm; thus it was in the target range. Compared to PM samples, it can be observed that in all three cases, the polymers could prevent the adhesion of the LAM particles, while the particle size of the polymers and LAM decreased due to co-milling.

Table 2. The particle size of PM and co-milled samples

	Length (µm)	Width (µm)	Average particle size of LAM (µm)
PM_LAM_PVP	26.80	18.96	7.63 ± 20.07
R_LAM_PVP	13.60	9.06	0.121 ± 0.027
PM_LAM_PVA	26.90	17.05	13.88 ± 21.15
R_LAM_PVA	25.33	16.61	0.15 ± 0.042
PM_LAM_NaHA	37.07	18.75	26.17 ± 27.68
R_LAM_NaHA	16.83	11.30	0.23 ± 0.016

4.1.3. Dissolution and permeability tests of the samples

In the case of the *in vitro* (Figure 2.) investigations, it can be generally said that the co-milled samples performed much better than the PMs. This means that higher dissolution (Figure 2. A) and permeability rates (Figure 2. B) were detected during the tests when LAM was co-milled with adjuvants. Especially the sample, which contained PVA, showed high values (100% released LAM after 5 mins and more than 50 µg/cm^2 concentration in the acceptor phase after 30 mins) in both experiments.

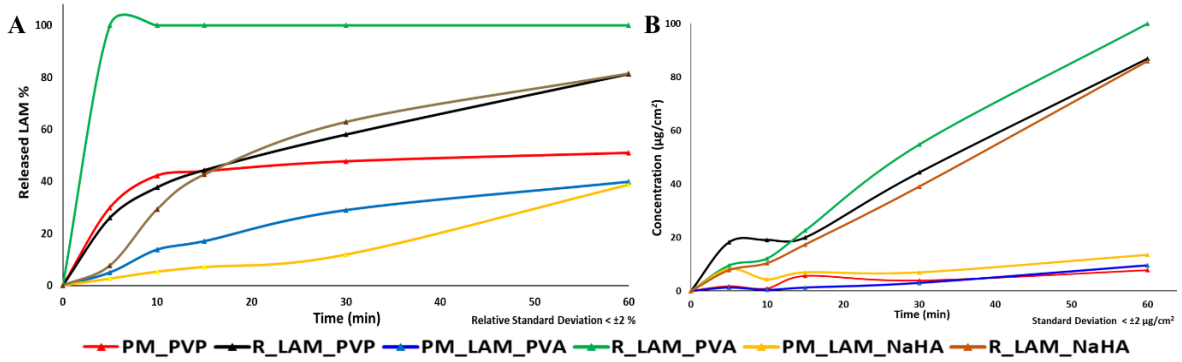

Figure 2. Dissolution curves (A) and permeability profiles (B) of the samples

4.1.4. Estimation and validation of the Design Space

Our aim afterward was to identify the DS by optimizing the parameters of the dry milling method by creating a more economical, time- and cost-effective sample preparation method. The major objectives of QbD are risk minimization and DS development for the product. The edges of the hypercube were between 1.4 h and 1.7 h in the case of milling time, 0.77 and 0.83 in the case of the PVA: LAM ratio, respectively. Milling speed was kept constantly 400 rpm. The parameters of the robust setpoint were 1.5 h, 400 rpm, and 0.8 PVA: LAM ratio (Figure 3.).

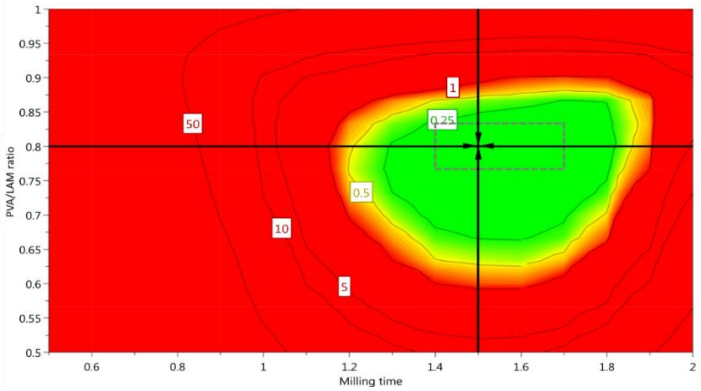

Figure 3. The Design Space of the dry milling method.

During the validation process, the P7 (nanoLAMpowder) sample showed the best values in this case too with a very small average particle size of LAM (97.46 nm). This small particle size, the homogenous distribution and the non-aggregated property of the LAM particles caused that the API left the surface of the polymer easier, which resulted in the best dissolution rate (97.32±4.95% released LAM after 10 mins) among the tested samples. The results of validation can be seen in Table 3.

Table 3. The results of particle size analysis

Sample	Y1- Mean size of LAM (nm)	Y2- Standard Deviation (± nm)
Powder 1 (P1)	521.42	310
Powder 2 (P2)	212.77	150
Powder 3 (P3)	198.71	120
Powder 4 (P4)	143.36	80
Powder 5 (P5)	124.18	60
Powder 6 (P6)	140.62	70
Powder 7 (nanoLAMpowder)	97.46	60

4.1.5. In vivo studies

The nanoLAMpowder formulation was compared to the PM and IV injection. The application of nanoLAMpowder sample resulted in a significantly higher drug concentration (2.16 ± 0.21 µg/brain g) in brain tissues compared to the PM (0.18 ± 0.76 µg/ brain g), which can be seen in Figure 4. In terms of cerebral AUC values of the formulations, the administration of IV injection resulted in a higher AUC value (253.60±7.66 µg/brain g*min) compared to the nasal formulations. However, LAM could reach the brain directly by axonal transport in the case of nanoLAMpowder (69.05±10.08 µg/ brain g*min), resulting higher AUC values than with the usage of PM (54.01±15.39 µg/brain g*min).

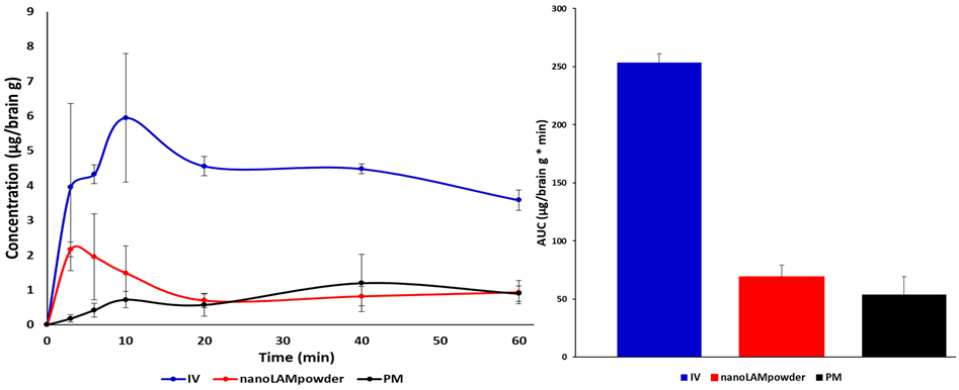

Figure 4. The concentration and AUC values of LAM in the brain samples

4.1.6. Stability study of the nanoLAMpowder

In the tested period, the key properties related to the powder formulation did not change considerably. The results of particle size determination resulted in same particle size of the product during the examined period (Table 4.), which shows that the product's particles did not aggregate. The size falls into the range which is desired in the case of nasal powders, which is 10-40 µm.

Table 4. The results of particle size investigation the product. In the table n.s means that there is no significant difference at 95% level.

	Average size of the product (µm)	t-value	p-value	Significance
1-day	29.91±15.85	-0.1435	0.8883	n.s.
3 months	28.48±12.81	0.2994	0.7690	n.s.
6 months	26.52±11.14	0.9064	0.3801	n.s.

The particle size of LAM in the formulation showed an increase with relatively high standard deviation (Table 5.). However, according to results of the statistical analysis there is no significant difference in the particle sizes during the storage period, thus the previously experienced rapid and high degree of release was predicted.

Table 5. The results of particle size investigation of LAM. In the table n.s means that there is no significant difference at 95% level.

	Average size of LAM (nm)	t-value	p-value	Significance
1-day	97±60	1.2382	0.2347	n.s.
3 months	105±77	0.7934	0.4408	n.s.
6 months	120± 84	-0.0408	0.9687	n.s.

The results of the structural investigatios (DSC and XRPD) confirmed that the LAM remained partly amorphous in the tested period. Moreover, according to the results of *in vitro* dissolution tests the rapid release of LAM was maintained during the examined period.

4.2. Development of NC formulations

4.2.1. Particle size, particle size distribution and surface charge characterization of NCs

The NCs were always in the 290-380 nm range that is acceptable according to the FDA regulatory, as the particle size of nanosystems has to be between 100-1000 nm. Our aim was to develop NCs that were in the lower part of this range and showed a homogenous particle

size population (PDI <0.2). These requirements were fulfilled for the NCs only if the liquid lipid: surfactant ratio was 1:1. LAM incorporation resulted in a significant increase in particle size as compared to blank NCs. In all cases, zeta potential values were similar, positive and close to zero that may be advantageous for mucoadhesion and mucodiffusion. The freeze-dried formulation showed some increase in particle size and PDI after redispersion (504±3 nm, 0.538 PDI), indicating some aggregation, that could happen due to the presence of mannitol. However, this aggregation was not observed in the freeze-dried state when the powder cake was analyzed with imaging technology as the particle size showed 179±62 nm. The results can be seen in Table 6.

Table 6. Results of the particle size and surface characterization of the NCs.

	Z-average (d. nm)	**PDI**	**Zeta potential (mV)**
Blank NCs after centrifugation (2:1 ratio)	2815 ± 159	0.795	0.99 ± 0.4
LAM NCs after centrifugation (2:1 ratio)	1210 ± 68	0.773	1.3 ± 0.1
Blank NCs after centrifugation (1:2 ratio)	1477 ± 72	0.643	0.80 ± 0.3
LAM NCs after centrifugation (1:2 ratio)	1399 ± 59	0.950	0.94 ± 0.5
Blank NCs after centrifugation (1:1 ratio)	294 ± 9	0.175	0.39 ± 0.2
LAM NCs after centrifugation (1:1 ratio)	305 ± 7	0.188	1.0 ± 0.3
FDNCs	Freeze-dried: 179 ± 62 After redispergation: 504 ± 3	0.538	26.5 ± 0.9

4.2.2. In vitro drug release and permeability studies

The *in vitro* release study showed faster release of LAM in the case of both NCs formulations compared to pure LAM powder (Figure 5. A). It was detected that more than 20% of LAM released after 5 min and ~60 % LAM after 15 min; afterward, a drug release plateau was observed. The FDNCs released the drug slower than NCs but markedly faster than the drug powder. In this case, ~40% LAM was released after 10 min, and 50% after 15 min, a point where the release started to level-off. At 15 min, both NCs formulations released between 2.5 and 3-fold more LAM than the drug powder. Next, we performed a permeability study to compare how the different formulations could modify the capacity of LAM for crossing biological barriers (Figure 5. B). NCs and FDNCs formulations performed similarly well in this experiment, and much better than a LAM powder, which achieved the lowest amount of permeated drug. In the case of the NCs formulations, ~25 $\mu g/cm^2$ LAM diffused through the membrane, which was 2.5 times higher than the amount of drug diffused from the raw powder formulation.

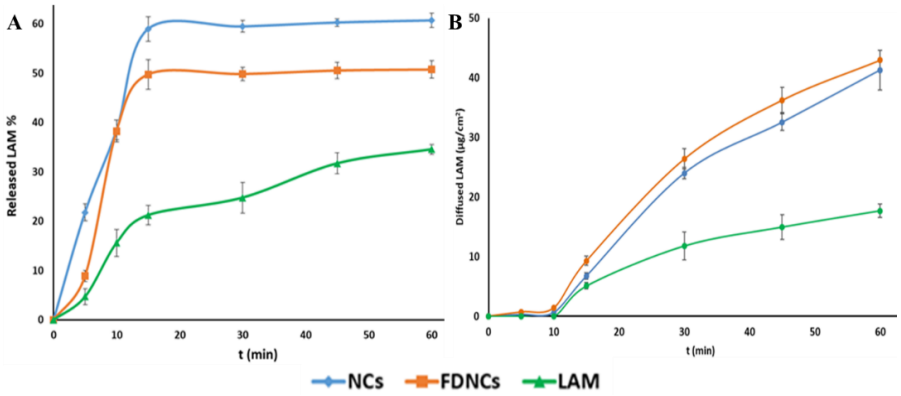

Figure 5. *In vitro* drug release (A) and permeability (B) studies of different NCs formulations and LAM powder

4.2.3. In vivo studies

Nasal administration of LAM in NCs achieved higher brain drug concentrations than FDNCs (Figure 6.). Indeed, the administration NCs resulted in significantly higher AUC values (11.65 ± 1.03 µg/ g*min) than FDNCs (2.06 ± 1.10 µg/g*min), while the AUC value of IV administration was 250.603 ± 7.66 min*µg/brain g. The ratio of AUC values between the liquid and the solid phase NCs was 5.65, which means that this formulation was capable of providing better drug absorption into the CNS.

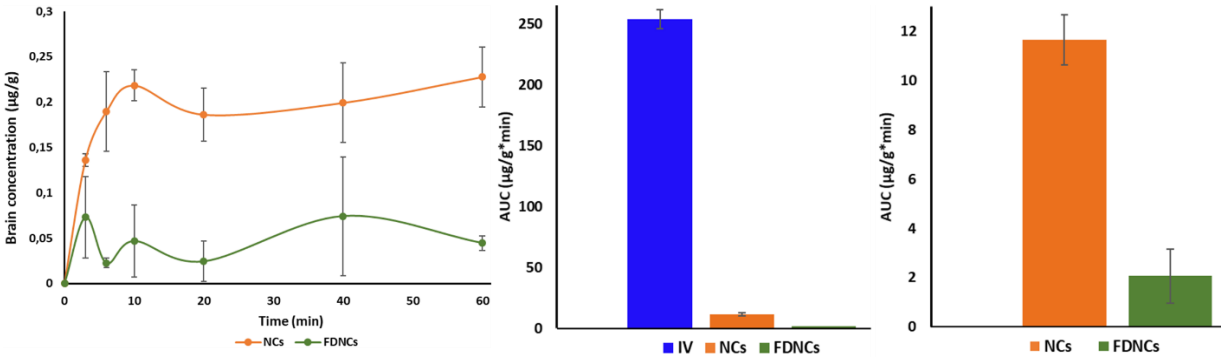

Figure 6. The concentration and AUC values of LAM in the brain samples.

4.3.Comparison of NP and NC formulations based on their *in vivo* performance

Table 7. summarizes the main *in vivo* properties of the samples. It can be seen that the nanoLAMpowder presented the highest amount in brain tissues and also in the plasma. Generally, compared to the NP samples, the NC formulations showed smaller AUC values. Moreover, the targeting of LAM into the CNS was much better in the case of NPs which can be seen from AUC_{brain}/AUC_{blood} data. The DTE values of NPs were also higher compared to the NCs, which means that LAM could reach its site of effect more efficiently when administered in powder formulation. The differences can be explained by the administered dose which is much higher in the case of NPs because the drug loading capacity of NCs is limited. Accordingly, the application of NP formulations can be advantageous because of the higher dose that can be administered into the nostril.

Table 7. Calculated parameters of the formulations.

	AUC_{brain} (µg/ g*min)	AUC_{blood}(µg/mL*min)	AUC_{brain}/AUC_{blood}	DTE
PM	54.01±15.39	8.59±1.35	6.29	3.11
nanoLAM powder	69.05±10.08	13.63±1.95	5.06	2.49
NCs	11.65±1.02	6.13±0.52	1.90	0.94
FDNCs	2.06±1.10	0.50±0.16	4.13	2.04

5. CONCLUSION

To summarize the thesis, the following statements can be made according to the aims:

- ✓ After the literature review, it was found that the combination of nasal drug delivery, nanosystems and QbD methodology can be advantageous to develop innovative and improved bioavailability products for LAM. The development of alternative dosage forms for LAM is important because it was found in the literature that on the market, only tablets, disintegrating tablets, and chewable tablets can be found, which can be ineffective in some cases, such as in malabsorption or acute diarrhea. It was also found that there is great potential –despite the poor number of marketed products- in the research and development of NPs, because they have many advantageous properties compared to liquid formulations. Moreover, the development of NCs can eliminate some possible drawbacks of nasal delivery according to the current information found in the literature.
- ✓ The quality influencing factors of NPs were collected with the help of an Ishikawa diagram. The results of the RA showed that amongst the CQAs, the particle size, its distribution and the dissolution rate of LAM, while amongst the CPPs, LAM: additive ratio, the milling time and speed are the most critical factors of NP production. After the preliminary studies, based on the results of the particle size analysis, *in vitro* release and permeability tests, PVA was chosen to serve as a matrix for nanosized LAM particles if the powder is prepared by co-milling. Optimization of the dry milling process was executed with DoE, which meant the Design Space determination and validation of the powder production. Due to the DS estimation, a more economical – time and material effective – sample preparation method was set up.
- ✓ NanoLAMpowder was tested in further investigations and was compared to its PM. The micrometric investigations showed that the LAM was in the nano range (97±60 nm) in the case of nanoLAMpowder, while it was aggregated in the PM sample. The results of the *in vitro* dissolution and permeability tests showed a rapid and high release from the nanoLAMpowder. The *in vivo* investigation showed that the plasma concentration of LAM was significantly higher in the IV group during the test compared to the nasally administered samples, where there was no considerable difference. The axonal transport of the drug was assumable by both intranasal formulations, but the brain AUC value of nanoLAMpowder

(69.05±10.08 μg/ brain g*min) was higher. According to the results of the stability studies, the properties (particle size of the product, crystallinity properties, *in vitro* release profile) of the NP did not change considerably after 6 months, so the efficiency presumably maintained during the tested period. Another aim was to develop and validate an inline horizontal penetration method, thus the analytical and investigation method was set up for LAM. We were able to develop an inline, modified diffusion method, which is a more accurate, easier to implement, more informative that has made our measurements more simple and efficient.

- ✓ Then, the aim was to develop and investigate novel, LAM containing core-shell NCs. The identification and prioritization of most influencing factors were collected with the help of an Ishikawa diagram. The components of the NC formulation were determined. According to the results of the preliminary experiments and size optimization, chitosan-coated NCs with LAM were formulated both as a liquid suspension and as a freeze-dried powder.
- ✓ The following step was the optimization of the production influencing the main factors. Accordingly, the parameters of freeze-drying were set and the most promising cryoprotectant was determined, which was mannitol. LAM released quickly from both NCs formulations. The permeation rate of LAM was also higher for the NC samples than for LAM in powder form. *In vivo* studies showed that LAM could reach the brain tissues in significant amounts, particularly in the case of NCs. The kinetics and biodistribution ratio of the drug between the brain a plasma suggest that there is axonal transport involved in drug absorption, which means that the LAM can reach its site of action in an amount sufficient for effect.
- ✓ In the living body, nanosystems could be more effective since their smaller particle size leads to a larger specific surface, which usually results in a faster dissolution rate and increased adhesion to the mucous membrane. The small particles in the nanoLAMpowder could leave the water-soluble PVA matrix easy, which resulted in great *in vitro* and *in vivo* performances. As for the NCs, chitosan was served as a shell component for the nanoparticles. This coating material can extend the residence time on the nasal mucosa and can improve the bioadhesion to the mucosa and in our case resulted in great API presence in the CNS, where it can block the voltage-related sodium channels.
- ✓ According to the *in vivo* results of the NP and NC formulations, it can be concluded that the presence of LAM in CNS was significantly higher in the case of NPs and also, the targeting of LAM was better in this case. In the case of NCs, it is not possible to encapsulate large amounts of API into the capsules, which results in lower administration doses. The administration dose of NPs is much higher, which in our case, resulted in better *in vivo* performance. Therefore, and because of the much more simple, economical and ecological

production method, in our opinion, the NPs could provide a great, short-term alternative for the marketed tablets in the therapy of epilepsy, if their application is not possible.

New findings of the work:

- ✓ As there is a limited number of nasal powder products on the market, the availability of information about them is poor despite, there is a great potential in their application. Their stability is better, the administration dose is higher compared to the liquid formulation, the residence time on mucosal surfaces is longer, fewer additives are needed during the formulation process. Also, these systems are special because their design, development, and optimization should be parallel with those of the delivery device to assure proper dosing. Thus, the thesis provides up-to-date and summarized knowledge about NPs.
- ✓ The determination of QTPP of nasal powder products and the collection of the most influencing CQAs (particle size, particle distribution and dissolution rate of LAM) and CPPs (milling time, milling speed, API: additive ratio). The dry milling sample production method was validated. Accordingly, a novel, partially amorphous, nanosized LAM containing, brain targeted NP was developed, which could provide an alternative for tablets when their administration is not possible. The formulation is necessary because there are no other LAM containing dosage forms on the market, only *per os* formulations. The LAM could release from the formulation rapidly and in a high amount.
- ✓ The adaption, development, and validation of a modified permeability investigation method. The modified diffusion model is suitable for inline, real-time detection, adapted to nasal conditions, using small volumes of phases, appropriately impregnated membrane, to monitor the diffusion of the drug, and to determine its concentration in the acceptor and donor phases. Accordingly, we have succeeded in developing an easy-to-implement, easy-to-reproduce, accurate, informative and time-efficient method.
- ✓ The development of a LAM containing, nanostructured NC formulation for nasal administration. Optimized technological protocol to prepare an intermediate sample containing NCs. The formulation can be administered in liquid and solid forms. The NC formulation can offer a great alternative for LAM administration into the CNS in a considerably high amount. Using this kind of nanoformulation, the advantages of nanosystems and nasal delivery can be combined.
- ✓ The developed formulations can be suitable-especially the NP formulation- for application in the therapy of epilepsy for a short time instead of tablets, for example in GI malabsorption or acute diarrhea, after further investigations.

PUBLICATIONS RELATED TO THE SUBJECT OF THE THESIS

1. **Gieszinger P.**, Bartos Cs., Szabó-Révész P., Ambrus R.- Nazális készítmények aktualitásai; bevitelre alkalmas eszközök és modern szerelékek. GYÓGYSZERÉSZET 61:(4) pp. 204-211. (2017)

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2. **Gieszinger P.**, Csoka I., Pallagi E., Katona G., Jojart-Laczkovich O., Szabó-Révész P., Ambrus R.- Preliminary study of nanonized lamotrigine containing products for nasal powder formulation. DRUG DESIGN DEVELOPMENT AND THERAPY 11: pp. 2453-2466. (2017)– Q1 journal

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5. **Gieszinger P.**, Csaba S. N., Garcia-Fuentes M., Prasanna M., Szabó-Révész P., Ambrus R.- Preparation and characterization of lamotrigine containing nanocapsules for nasal administration. EUROPEAN JOURNAL OF PHARMACEUTICS AND BIOPHARMACEUTICS. Under review. – Q1 journal

6. **Gieszinger P.**, Katona G. Szabó-Révész P., Ambrus R..- Stability study of nasal powder formulation containing nanosized lamotrigine. ACTA PHARMACEUTICA HUNGARICA. Under review. - Q4 journal

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Oral presentations

1. **Gieszinger P.-** Ko-őrléssel előállított, nanonizált lamotrigint tartalmazó nazális gyógyszerforma vizsgálata. XII. Clauder Ottó emlékverseny, Budapest (2016)

2. **Gieszinger P.-** Nanonizált hatóanyag tartalmú nazális por előállításának optimalizálása. Szegedi Tudományegyetem Sófi József a Szegedi Tehetségekért Alapítvány Ösztöndíj Konferencia, Szeged (2018)

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4. **Gieszinger P**., Ambrus R., Szabó-Révész P. - Nasal formulation of active ingredients to induce systemic and central nervous systemic effects. I. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, Szeged (2019) pp. 22-22., 1 p.

5. **Gieszinger P.**, Csaba S. N., Garcia-Fuentes M., Prasanna M.; Katona G., Szabó-Révész P., Ambrus R. - Lamotrigin tartalmú nanokapszulák fejlesztése. Gyógyszertechnológiai és Ipari Gyógyszerészeti Konferencia: A Magyar Gyógyszerésztudományi Társaság Gyógyszeripari Szervezetének és Gyógyszertechnológiai Szakosztályának Konferenciája. Siófok, (2019) pp. 22-22., 1 p. 2019.

6. **Gieszinger P**., Ambrus R., Szabó-Révész P.- Formulation of nasal drug delivery systems to induce systemic and central nervous system effect. II. Symposium of Young Researchers on Pharmaceutical Technology, Biotechnology and Regulatory Science, Szeged (2020), pp. 8-8., 1 p.

Poster presentations

1. Ambrus R.*, **Gieszinger P**., Pallagi E., Csóka I., Szabó-Révész P.- Formulation of a nasal powder containing nanonized antiepileptic Lamotrigine, by applying the QbD approach. 11th Central European Symposium on Pharmaceutical Technology 2016, Belgrade, Serbia

2. **Gieszinger P**.*, Casian T., Tomuta I., Szabó-Révész P., Ambrus R.- Development of a nasal powder preformulation process by Design of Experiment method. ACTA PHARMACEUTICA HUNGARICA 87:(043) Paper P2B-4. 1 p. 7th BBBB International Conference on Pharmaceutical Sciences. 2017, Balatonfüred, Hungary

3. Ambrus R., **Gieszinger P**., Szabó-Révész P., Sztojkov-Ivanov A., Ducza E., Márki Á., Gáspár R., Kecskeméti G., Janáky T., Bartos Cs.- *In vitro* and *in vivo* characterization of nasal powder containing nanonized lamotrigine. 12th Central European Symposium on Pharmaceutical Technology and Regulatory Affairs 20-22.09. 2018, Szeged, Hungary

4. **Gieszinger P.**, Csaba S. N., Garcia-Fuentes M., Prasanna M., Szabó-Révész P., Ambrus R.- Preparation and characterization of lamotrigine containing nanocapsules for nasal administration. 3rd European Conference on Pharmaceutics - Bringing science into pharmaceutical practice p. 21. 2019, Bologna, Italy

5. **Gieszinger P.**, Szabó-Révész P., Ambrus R.- Nanonizált lamotrigin tartalmú nazális porok stabilitásvizsgálata. Congressus Pharmaceuticus Hungaricus XVI. 2020, Debrecen, Hungary

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