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PII: S0928-0987(19)30372-0
DOI: https://doi.org/10.1016/j.ejps.2019.105099
Reference: PHASCI 105099

To appear in: *European Journal of Pharmaceutical Sciences*

Received date: 15 July 2019
Revised date: 10 September 2019
Accepted date: 1 October 2019

Please cite this article as: Patrícia C. Pires , Diana Peixoto , Isaura Teixeira , Márcio Rodrigues , Gilberto Alves , Adriana O. Santos , Nanoemulsions and thermosensitive nanoemulgels of phenytoin and fosphenytoin for intranasal administration: formulation development and in vitro characterization, *European Journal of Pharmaceutical Sciences* (2019), doi: https://doi.org/10.1016/j.ejps.2019.105099

Nanoemulsions and thermosensitive nanoemulgels of phenytoin and fosphenytoin for intranasal administration: formulation development and *in vitro* characterization

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Abstract

Phenytoin is a low solubility anticonvulsant drug. It has, nonetheless, other possible therapeutic indications, such as neuropathic pain, including trigeminal neuralgia, or wound healing. Its use has decreased due to side effects, but nasal/intranasal administration could significantly increase drug safety and efficacy.

The aim of this work was to develop and study nanoemulsions and thermosensitive nanoemulgels of phenytoin and fosphenytoin, in combination, for intranasal administration, with immediate and sustained release profiles.

Nanoemulsions were prepared by adding the aqueous phase, containing gelling polymers in the case of nanoemulgels, to emulsion preconcentrates, followed, in the optimized procedure, by premix membrane emulsification. Formulation design and optimization was guided by drug strength, rheological behavior, osmolality, mean droplet size and polydispersity.

Fosphenytoin interfered significantly with Carbopol but not with Pluronic's gelation, and allowed to achieve drug strengths equivalent to 22 or 27 mg/g of phenytoin in lead nanoemulsions, and 16.7 mg/g of phenytoin in the lead nanoemulgel. The final selected low viscosity nanoemulsions had an immediate or prolonged fosphenytoin release profile, depending of anhydrous phase proportion (10% or 40%, respectively). The thermosensitive nanoemulgel, with 10% anhydrous phase, showed prolonged drug release. Future studies will establish whether they are more suited for topical effects or therapeutic brain delivery.

Keywords: Intranasal, Nanoemulgel, Nanoemulsion, Phenytoin, Fosphenytoin, Premix membrane emulsification.

Abbreviations: CV – coefficient of variation; FOS – fosphenytoin; FPT – fosphenytoin, phenytoin and Transcutol; FT – fosphenytoin and Transcutol; HPLC – high performance liquid chromatography; LLOQ – lower limit of quantification; N – nanoemulsion; P – Pluronic; PC – Pluronic and Carbopol; PDI – polydispersity index; PHT – phenytoin; QC – quality control; SD – standard deviation; SEEDS – self-emulsifying drug delivery system; T_aMax – maximum acceleration temperature; TE – thermosensitive emulgel; TG – thermosensitive gel; T_{gel}50 – half-gelation temperature; TN – thermosensitive nanoemulgel; USP – United States Pharmacopeia.

1. Introduction

Convulsive *status epilepticus* is the most severe manifestation of epilepsy. It is associated with a high degree of morbidity and, in some cases, even mortality, which can reach up to 30% in adults. Today, the first-line treatment in hospital setting is an intravenous benzodiazepine. But although it has fallen out of use as a first-choice treatment, due to lower safety, phenytoin (or its more soluble prodrug fosphenytoin) is still used as intravenous second-line therapy (Glauser et al., 2016). Phenytoin is also still widely used in oral form for the chronic treatment of epilepsy (World Health Organization, 2017). Furthermore, there are very few studies directly comparing phenytoin to other antiepileptics, and the ones that are robust enough usually conclude non-inferiority, which means that although it has a higher associated risk due to systemic side effects, it seems to be equally effective (Glauser et al., 2016). In addition, phenytoin has other established or potential therapeutic applications, as it is approved as an antiarrhythmic and has been explored throughout the years in neuroprotection, retinoprotection, breast cancer, depression, bipolar disorder and wound healing (Bartollino et al., 2018; Borowicz and Banach, 2014; Hesselink and Kopsky, 2017; Hesselink, 2017). That being said, by using strategies that could reduce peripheral systemic side effects (cardiovascular complications, liver toxicity, osteopenia, peripheral neuropathy), such as local or targeted delivery to the intended sites, phenytoin could become a drug of great interest once more (Poplawska et al., 2015; Shih et al., 2016).

One approach for partially targeted brain transport, minimizing systemic distribution in the periphery, is the use of the nasal route of administration, where the drugs can undergo neuronal transport to the brain through the olfactory nerves (Pires and Santos, 2018). It is also an interesting alternative to both the intravenous and oral routes for being non-invasive and avoiding first-pass metabolism (Djupesland et al., 2014; Kammona and Kiparissides, 2012). This route has had an increase in attention over the past decades to deliver drugs for the treatment of neurodegenerative and psychiatric diseases, and there are even several studies regarding the intranasal delivery of other antiepileptics (Kapoor et al., 2016). In fact, intranasal benzodiazepines have long shown to be at least as effective as their intravenous counterparts (Zaccara et al., 2017). Intranasal midazolam (off-label use) has even been recommended as first-line therapy in prehospital setting by the American treatment guidelines (Glauser et al., 2016) and more recently a nasal preparation received marketing

approval in the United States of America, with the brand name Nayzilam® (U.S. Food and Drug Administration, n.d.). Intranasal drug administration may require either potent drugs (like benzodiazepines) or very high drug strength formulations, due to the reduced volume of administration, which is a hurdle in the case of phenytoin (Wermeling, 2009). Intranasal administration of phenytoin could, however, have other applications requiring lower drug strength, like postsurgical nasal wound healing or trigeminal neuralgia (Hesselink, 2017; Simsek et al., 2014).

Given the very low water solubility of phenytoin, one way of formulating it would be into a nanosystem, more specifically an oil-in-water nanoemulsion, where the drug would be solubilized in the internal phase. In general, nanosystems can protect drugs from metabolic or chemical degradation, reduce protein binding and increase overall diffusion through biological membranes (Pires and Santos, 2018). Nanoemulsions are a specific nanosystem category that has several advantages, including high kinetic stability, and certain formulas emulsify spontaneously just by adding the aqueous phase component to the mixture of oil and surfactants in the right proportions, therefore being easy to prepare (Kumar et al., 2016). Alternatively, phenytoin's hydrophilic prodrug, fosphenytoin, solves phenytoin's issue of water insolubility, but its anionic nature makes it less prone to passive absorption. However, Antunes Viegas et al. (2016) demonstrated the presence of phosphatase activity within the nasal mucosa, which promotes *in situ* bioconversion of fosphenytoin to phenytoin. Furthermore, fosphenytoin could partially permeate porcine nasal mucosa *ex vivo* as prodrug, in addition to the permeation of the neutral parent drug phenytoin formed by *in situ* bioconversion.

Moreover, as mucociliary clearance does not allow much time for drug absorption to occur, the addition of a mucoadhesive and/or thermosensitive polymer to the external phase of the nanoemulsion could help increase its retention in the nasal cavity, the first by allowing adhesion to the nasal mucosa, and the second by increasing the fomulation's viscosity when heated, potentially leading to higher bioavailability. Thermosensitive polymers, when in solution at a sufficient concentration, have the ability to undergo sol-gel phase transition with temperature increase. Such is the case of poloxamer 407 (brand name Pluronic® F-127), an amphiphilic block copolymer whose gel formation is associated with micellization, and that has been extensively used in the development of *in situ* nasal gels. Nasal formulations associating poloxamers with carbomers, which combine viscosity increase and mucoadhesive properties, have also been reported in the scientific literature (Karavasili and Fatouros, 2016; Rowe et al., 2009). These gel forming polymers can be used in the preparation of emulgels, a combination of emulsions and gels, hence combining the properties of both: increased stability and drug solubility (emulsions) and increased viscosity with potentially enhanced retention times (gels).

Thus, the main aim of this work was to develop liquid formulations of phenytoin in a soluble form, suitable for intranasal administration, one with a faster release profile, and another with a prolonged release profile, promoting the preparation's retention in the nasal cavity. The chosen formulation strategy was to develop a liquid nanoemulsion of phenytoin, which could be prepared by self-emulsification upon mixture of anhydrous and aqueous phases, and derive a second formulation in the form of a thermosensitive nanoemulgel for increased retention and sustained drug release. At

the same time, we aimed to gain understanding about formulation factors' influence in size dispersion, viscosity and gelation temperature of phenytoin nanoemulsions and nanoemulgels.

2. Materials and methods

2.1 Materials

The acid form of phenytoin used in formulation development was purchased from Acros Organics (Geel, Belgium), whilst the United States Pharmacopeia (USP) reference standard, for high-performance liquid chromatography (HPLC), was bought from Sigma-Aldrich (Steinheim, Germany). Fosphenytoin used in formulations was a gift sample from JPN Pharma (Mumbai, India), provided as a di-sodium salt, but mass concentration in the text will be indicated as calculated for the acid form. The fosphenytoin USP reference standard was acquired from Sigma-Aldrich (Steinheim, Germany). HPLC grade methanol, analytical grade trimethylamine, sodium chloride and sodium hydrogen carbonate were bought from Fisher Scientific (Leicestershire, United Kingdom). Dibasic sodium phosphate was bought from Acros Organics (Geel, Belgium) and monobasic sodium phosphate from Sigma-Aldrich (Steinheim, Germany). Calcium chloride was acquired from Panreac (Barcelona, Spain). Potassium chloride was bought from Chem-Lab (Zedelgem, Belgium). Magnesium chloride and sodium hydroxide were acquired from Labkem (Barcelona, Spain). The carbomer (Carbopol® 971P) was donated by Lubrizol (Brussels, Belgium). The oil, a medium-chain triglyceride (Miglyol® 812), the hydrophilic surfactant polysorbate 80 (Tween 80) and the cosolvent diethylene glycol monoethyl ether (Transcutol® P) were all acquired from Acofarma® (Barcelona, Spain). Hydrochloric acid was bought from Fluka (Seelze, Germany). The poloxamer (Pluronic® F- 127) was acquired from Sigma-Aldrich (Steinheim, Germany). For simplification, excipients will simply be referred to in the text by their common brand name. Water was always of ultra-pure grade (Milli-Q water apparatus, 0.22 μm filter, Merck, Darmstadt, Germany).

2.2. Preparation of nanoemulsions and thermosensitive gels

Emulsion preconcentrates were prepared by weighing together the oil (Miglyol 812), surfactant (Tween 80), and cosolvent (Transcutol P). Phenytoin was dissolved in this mixture before emulsification (Figure 1, step a.). Aqueous phase was made of either water, a fosphenytoin aqueous solution in which pH was adjusted to near 7 (Orion Star A211 pH meter, Thermo Fisher Scientific, Massachusetts, United States of America), or a thermosensitive gel, either way guarantying that the pH of all formulations was close to neutrality. Thermosensitive gels of Pluronic and Carbopol (TG_{PC} and TG_{PC+FOS}) were prepared by dispersing Carbopol 971P in water or fosphenytoin solution, adjusting pH to 6.5 - 7.0, followed by the addition of Pluronic F-127, final mass adjustment, and overnight agitation at 4 ºC. A thermosensitive gel with no Carbopol (TG_{P+FOS}) was also prepared, with only Pluronic and fosphenytoin in its composition.

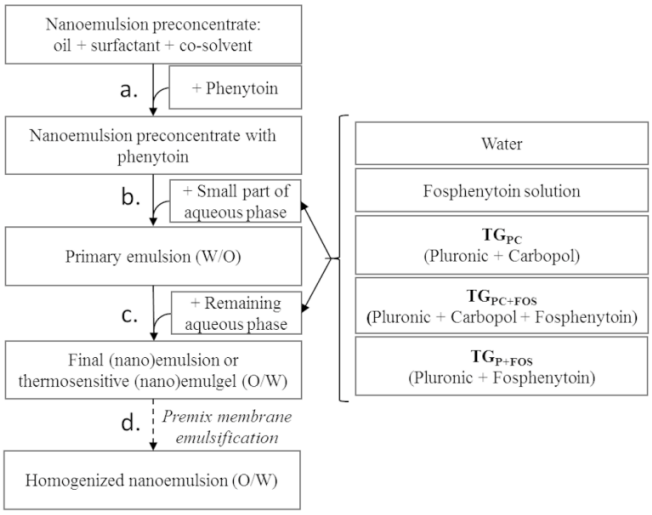

Figure 1. Flow chart of emulsions' preparation. Letters a., b., c. and d. represent procedural steps, explained in the text. FOS – fosphenytoin; O/W – oil-in-water emulsion; P – Pluronic; PC – Pluronic and Carbopol; TG – thermosensitive gel; W/O - water-in-oil emulsion.

Emulsions were prepared by weighing the preconcentrate and adding a small part of the aqueous phase in order to create a water-in-oil emulsion (Figure 1, step b.), with mild manual or magnetic agitation at room temperature or 15 ºC (in case of emulgels with low gelation temperature). The rest of the aqueous phase was then added to invert the emulsion (Figure 1, step c.), leading to an oil-in-water system, as desired.

In the final selected formulations - N_{FOS} 4:6, N_{FOS} 1:9, and TN_{P+FOS} 1:9, two nanoemulsions and one thermosensitive nanoemulgel (emulsion with a thermosensitive gel as the external phase) - after the formation of the emulsion, an additional homogenization was performed, by premix membrane emulsification (Figure 1, step d.). It consisted of mechanical extrusion, at room temperature, using a mini-extruder set (Avanti Polar Lipids, Alabama, United States of America), though a 0.2 µm pore size polycarbonate membrane (19 mm, Whatman® Nuclepore™ Track-Etched, Sigma-Aldrich, Steinheim, Germany). A summary of the composition of the most relevant formulations is shown in Table 1.

Table 1. Summary of the composition of the most relevant developed formulations.

Preconcentrate, final substance concentration (mg/g)	Aqueous phase	final concentration (expressed as

Formula name	Composition (w/w %, before emulsification)					% (w/w)		
	Miglyol 812	Tween 80	Transcutol	Phenytoin				
N_{FOS} 4:6	8	18.4	13.6	4	Fosphenytoin aqueous solution, 4.31%	60	25.9	22.0
N_{FOS} 1:9	2	4.6	3.4	1		90	38.8	27.0
TG_{PC}	-	-	-	-	Pluronic + Carbopol, 17% + 0.2%	100	-	-
TG_{PC+FOS}	-	-	-	-	Pluronic + Carbopol + Fosphenytoin, 17% + 0.2% + 2.23%	100	22.3	15.5
TE_{PC+FOS}	2	4.6	3.4	1		90	20.1	15.0
TG_{P+FOS}	-	-	-	-	Pluronic + Fosphenytoin, 16% + 2.5%	100	25.0	17.4
TN_{P+FOS} 4:6	8	18.4	13.6	4		60	15.0	14.4
TN_{P+FOS} 1:9	2	4.6	3.4	1		90	22.5	16.7

FOS – fosphenytoin; N – nanoemulsion; P – Pluronic; PC – Pluronic and Carbopol; TE – thermosensitive emulgel; TG – thermosensitive gel; TN – thermosensitive nanoemulgel.

2.3 Mean size, polydispersity index and zeta potential

Formulations that resulted from internal phase dispersion, in either vehicle or drug-loaded external phase, were characterized by droplets' mean hydrodynamic size (from now on simply referred to as mean size) and polydispersity index (PDI), obtained by cumulants' analysis of dynamic light scattering data, and zeta potential, determined by electrophoretic light scattering, using a Zetasizer Nano ZS apparatus (Malvern, United Kingdom). Samples were diluted 25- or 250-fold in water, and measured at 25 ºC in disposable ultra-violet/visible polymethyl methacrylate cuvettes (Kartell, Noviglio, Italy). For zeta potential measurements, a Dip Cell (ZEN 1002, Malvern, United Kingdom) was used. Analysis was performed automatically three times for each sample, in at least two different samples of each batch.

2.4 Osmolality and rheology

Osmolality was determined with a freezing point osmometer (Osmomat 3000, Gonotec, Berlin, Germany) and mean values were calculated using 3 to 5 measurements of each batch.

Viscosity measurements were made by means of a thermostated Brookfield DV3T cone-plate rheometer (Brookfield Ametek, Massachusetts, United States of America), using either CP40Z or CP52Z cones, and a sample volume of 0.5 mL.

In fluids with Newtonian rheological behavior, zero shear viscosity was considered to be the viscosity value measured at the highest torque (and consequently, having the lowest measurement error). When in the presence of non-Newtonian pseudoplastic behavior, zero shear viscosity was inferred from measurements at different shear rates and constant temperature: either 32 ºC, the minimal nasal cavity temperature; 20 ºC, average room temperature; or 15 ºC, when measuring at 20 ºC was not adequate to characterize the sol state because gelation temperature was too close. Gelation temperatures were determined at a constant shear rate (80 s^{-1}) and varying temperatures. Measured values that were not within the torque interval correspondent to a minimum of 95% measurement accuracy were not considered for analysis.

2.5 *In vitro* drug release assay

The *in vitro* drug release study was performed using horizontal Ussing Chambers (Harvard Apparatus, NaviCyte, Hugstetten, Germany), with temperature being kept at 32 ºC by a heating bath (Grant Instruments, Cambridge, England). Membranes were made of hydrophilic polyethersulfone and had a 0.2 µm pore size (Supor® membrane disc filters, Pall Life Sciences, Michigan, United States of America).

The bottom chamber was filled with 1.8 mL of nasal fluid simulant buffer, composed of: monobasic sodium phosphate (7 mM), dibasic sodium phosphate (3 mM), potassium chloride (30 mM), sodium chloride (107 mM), calcium chloride (1.5 mM), magnesium chloride (0.75 mM), and sodium hydrogen carbonate (5 mM) (salt composition and concentration adapted from the literature) (Burke, 2014; Grubb et al., 2002; Vanthanouvong et al., 2006). pH was adjusted to 6.5 (nasal pH), and each bottom chamber contained the same amount of Transcutol as that of the formulation that was being evaluated for release (either 3.4, 13.6 or 30.0% (w/w)), so that phenytoin's solubility would not be reduced, neither in the bottom chamber (phenytoin's solubility in buffer only would be very low), nor in the upper chamber (due to Transcutol diffusion from upper to bottom). When the chambers were fully mounted, 200 µL of this buffer was placed on the upper side of the membrane, and the chambers were let to stabilize for 1 hour in order to reach the intended temperature. Then, the buffer on the upper side of the membrane was removed and replaced with 200 µL of the formulation. Samples of 100 µL were taken from the bottom chamber at 5, 10, 20, 40, 60, 90, 120, 150, 180, 210 and 240 minutes, and the collected volume was replaced each time with buffer solution. Homogenization of the bottom chamber fluid was done through magnetic steering.

Collected samples were diluted in two-steps: the first dilution (10 fold) was done using the nasal fluid simulant buffer with Transcutol, and the second dilution (7 fold) was done using the same buffer but without the cosolvent. For the quantification of initial drug concentration in the formulations the second dilution was 70 fold. Drug quantification was then done by HPLC.

Aside from the selected nanoemulsions, a positive control of drug release was made with an aqueous solution of both phenytoin, at 0.2 mg/g, and fosphenytoin, at 1.3 mg/g, a 1 to 6.5 proportion (the same as in N_{FOS} 4:6), also containing Transcutol at 30% (w/w) to increase phenytoin solubility. In parallel, the equivalent fosphenytoin solutions (with and without Transcutol) were also evaluated.

2.6 High-performance liquid chromatography method validation

2.6.1 Chromatographic apparatus and conditions

Chromatographic apparatus consisted of a HPLC system (LC-2010A HT Liquid Chromatography) coupled with a diode-array detector (SPD-M20A), with instrumental parts being controlled automatically by the data acquisition software (LabSolutions, version 5.52), all bought from Shimadzu (Kyoto, Japan). Analyte separation was performed at 30 ºC on a reversed-phase guard column (C18, 5 µm particle size, 4 x 4 mm) connected to a reversed-phase column (C18, 3 µm particle size, 55 x 4 mm), LiChroCART® Purospher® STAR models, both purchased from Merck (Darmstadt, Germany). Elution was done at 1 mL/min in isocratic mode, and mobile phase was filtered (0.2 µm pore) and degassed (ultrasound, 30 minutes) prior to injection, being made of 36% methanol and 64% aqueous phase (sodium phosphate buffer, 10 mM, pH 3, with 0.25% triethylamine), v/v. Analyte detection was done at 215 nm for both fosphenytoin and phenytoin, with separation being achieved within 20 minutes of each run, and an injection volume of 20 µL.

2.6.2 Method validation

Method validation followed the Food and Drug Administration guideline criteria (FDA et al., 2018).

For the preparation of the calibration standards, individual stock solutions of fosphenytoin and phenytoin were made by dissolving the compounds in methanol, at 5 mg/mL. From these, intermediate combined solutions were prepared by spiking nasal simulant buffer, with 30% (w/w) Transcutol, with both drugs. The final dilution was made using the same buffer, but without the cosolvent, creating calibration standards with both drugs at 0.1, 0.2, 0.5, 1.0, 2.0, 5.0, 15.0, 25.0 or 40.0 µg/mL. The lower limit of quantification (LLOQ) was the lowest analyte concentration that could be quantified with inter/intraday precision and accuracy within the acceptance criteria. Quality control (QC) samples were prepared in the same way, but independently, having separate stock solutions. They comprised three different concentration levels, representing the low (QC_1), medium (QC_2) and high (QC_3) ranges of the calibration curves. Final concentrations were of 0.3, 20.0 and 36.0 µg/mL for both analytes. An additional sample was also prepared at the concentration of the lower limit of quantification (QC_{LLOQ}). When not in use, all stock solutions were kept at -80 ºC (U570 Premium ultra-low temperature freezer, Eppendorf, Hamburg, Germany) and all intermediate, calibration standards and QC solutions were kept at 4 ºC (Liebherr, Kirchdorf, Germany).

Linearity was assessed by preparing calibration curves for both analytes using the 9 defined calibration standards. It was evaluated on three different days (n = 3). The obtained data was then analyzed according to a previously developed mathematical method (Almeida et al., 2002), through a transformation by a weighted linear regression, using a specific function as a weighting factor – in this case, $1/y^2$. The calibration curves were then constructed by putting peak area as function of the corresponding nominal concentrations.

As for precision and accuracy, validation guidelines define that precision (% of coefficient of variation, CV) must be lower than or equal to 15% (or 20% for the LLOQ) and accuracy (% of bias) must be within ± 15% (or ± 20% for the LLOQ). Interday precision and accuracy were assessed for quality control samples on three consecutive days (n = 3), and intraday parameters were determined by analyzing five sets of samples on the same day (n = 5).

Method selectivity was evaluated by processing and analyzing blank samples (nasal simulant buffer with Transcutol) to determine whether matrix substances interfered with the retention times of the analytes. Formulation vehicles' interferences were also tested.

Absolute recoveries were calculated by comparing peak areas from QC_1, QC_2 and QC_3 samples to the correspondent aqueous solutions with the same nominal drug concentrations.

Short-term stability was evaluated for QC_1 and QC_3 samples, in replicate (n = 5). Stability samples were compared to previously analyzed QC samples that served as reference. In order to consider a given sample to be stable, the percentual deviation of the stability samples' peak area values in comparison with the reference had to be between 85 and 115%. Stability was evaluated at room temperature for 24 hours, which is the estimated maximum amount of time for which samples are kept in the auto-sampler before analysis. The effect of 24 hour freeze-thaw cycles was also assessed, by keeping samples at -20 ºC and doing 3 cycles of unfreezing/refreezing, on three consecutive days.

2.5 Data analysis

Formulation and drug release data analysis was performed using Prism software, version 6.0, from GraphPad, and the significance level was set at 0.05.

Zero shear viscosity of non-Newtonian fluids was calculated by fitting a linear regression model to the data and determining the zero of the function. Whenever required, a logarithmic transformation of the data was performed before regression analysis. Statistical significance of the differences in viscosity between formulations (zero shear for non-Newtonian behavior) and at different temperatures was evaluated, after a logarithmic transformation of the viscosity values, by two-way ANOVA analysis, followed by a Tukey's multiple comparisons post-test.

The calculation of the half-gelation temperature, defined as the temperature at which gelation is at 50% ($T_{gel}50$), was made by fitting the non-linear regression model *log (agonist) vs. response, variable slope, four parameters* to the viscosity vs temperature data. The significance of the difference between formulations' $T_{gel}50$ was assessed by an F test. The maximum acceleration temperature (T_aMax), considered as the temperature at which gelation starts, was calculated as the maximum of the second derivative of the function given by the obtained non-linear regression model.

A two-way ANOVA analysis, with a Sidak's multiple comparisons post-test was used to assess the statistical significance of differences in mean size and PDI between formulations, and also to compare between a different number of extrusion cycles in the same formulation.

For the calculation of the drug release rate, an adaptation of the Higuchi model was applied (Ramteke et al., 2014; United States Pharmacopeial Convention, 2017). Both time (X) and drug release percentage or accumulated drug quantity (Y) were transformed: X by calculating its square root ($X = \sqrt{X}$) and Y by dividing by the area of the membrane used in the assay ($Y = Y/0.64$). After transformation, a linear regression was applied, and late time points for which correspondent values fell out of the linear zone were excluded. The drug release rate corresponded to the slopes, which were compared two-by-two using an F test to assess whether they differed significantly between formulations.

4. Results and discussion

4.1 Phenytoin nanoemulsion development

The preliminary steps of phenytoin nanoemulsion development were taken in the context of a compounding formula for an oral self-emulsifying drug delivery system (SEEDS) of phenytoin. The lead formula published by Atef and Belmonte (2008) was modified by replacing most excipients for others that were more easily accessible and commonly used in community and hospital pharmacies in Portugal, like the oil Miglyol 812, the hydrophilic surfactant Tween 80, and the hydrophobic surfactant Span 80 (unpublished work, not shown). Excipient proportion was slightly adjusted as well, but Transcutol was kept at high percentage (55%).

For having a safe intranasal administration we desired a lower cosolvent amount than the used in the previously mentioned oral SEEDS, and isotonic to slightly hypertonic formulations (within the established limits for marketed nasal preparations) (Marx et al., 2015). By screening different levels of excipients, it was determined that, for maximum phenytoin solubilization in the formula, either Transcutol proportion should be > 0.25, or Tween 80 > 0.6 (Figure 2A). With simultaneous lower levels of both excipients the preconcentrates were unable to solubilize phenytoin, even at 15 mg/g. Furthermore, to avoid drug precipitation upon emulsification, Transcutol proportion should be in the range of approximately 0.2 to 0.35 (Figure 2B). Among the series of tested formulations (data not shown), one fulfilled these criteria (20% oil, 46% surfactant and 34% cosolvent), also originating a nanoemulsion at 10% (w/w) in water (203.5 ± 4.6 nm mean size, PDI of 0.316). Nevertheless, this preconcentrate could only incorporate phenytoin at 10 mg/g without the occurrence of precipitation upon emulsification. The osmolality of the obtained nanoemulsion was 384 mOsmol/kg. Although this is only slightly above the osmolality of plasma, it is in great part due to the presence of Transcutol in the formula, since this cosolvent partitions to the water phase. We could not find any information about the permeability of Transcutol through the nasal mucosa, but given its low molecular weight, neutrality, and miscibility with both hydrophilic and lipophilic environments, it is safe to expect that it will permeate, not contributing to the preparation's tonicity.

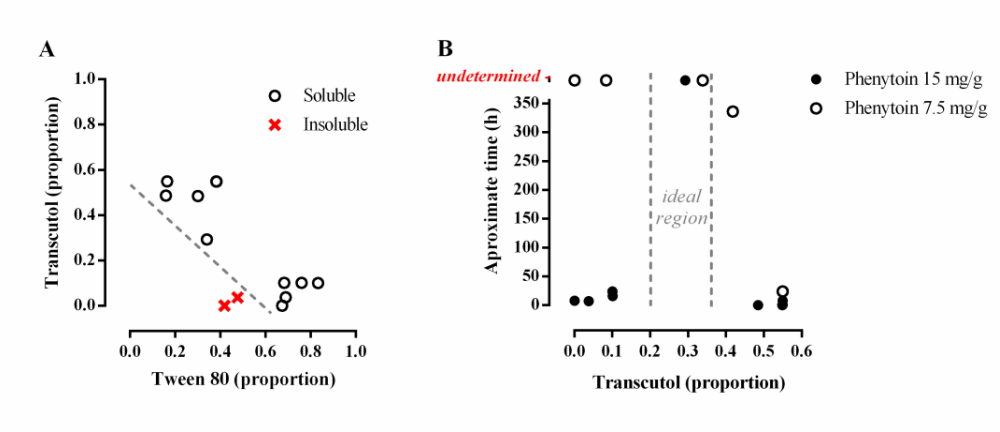

Figure 2. Intranasal nanoemulsion optimization. Transcutol *versus* Tween 80 proportions in oil formulations (A); time until phenytoin precipitation after emulsion formation with different Transcutol proportions in the anhydrous mixture (B). Shown values of phenytoin concentration are referring to those in the preconcentrate. Emulsions were prepared at 10% (w/w) of preconcentrate in water.

As it was only possible to incorporate 10 mg/g of phenytoin in the preconcentrate, and given that it was only 10% of the nanoemulsion, the drug concentration upon emulsification dropped to 1 mg/g (0.1%). This concentration is most likely too low to try to achieve therapeutic levels of phenytoin in the brain after intranasal administration, or even local effects, and therefore strategies to increase drug strength were needed.

Hence, a solution of the soluble prodrug fosphenytoin, at 43.1 mg/g (equivalent to 30 mg/g of phenytoin), was used as the aqueous phase at 90 or 60% (w/w), originating nanoemulsions with a phenytoin strength equivalent to 27 mg/g (N_{FOS} 1:9) or 22 mg/g (N_{FOS} 4:6), respectively, that did not form any precipitate after emulsification for at least 10 days. Although N_{FOS} 1:9 had a higher phenytoin strength equivalent, N_{FOS} 4:6 had more phenytoin in its active form (and hence less prodrug), therefore both formulations were considered to be potentially useful for further studies. The choice to associate fosphenytoin to phenytoin was supported by previous work, which demonstrated that both fosphenytoin permeation and conversion to the active form occur in the nasal mucosa, as referred to in section 1 (Antunes Viegas et al., 2016). While an initial dose of phenytoin is readily available for passive diffusion, in parallel more phenytoin will be generated *in situ* due to the conversion of the prodrug to the active form, and some permeation of fosphenytoin by alternative pathways will also happen.

The aqueous phase alone (fosphenytoin solution) was isotonic (283 mOsmol/kg). Nevertheless, the resulting emulsions were moderately to highly hyperosmotic (Table 2). However, Transcutol is the component that increases osmolality most meaningfully by diffusing to the aqueous phase, and it is not expected to contribute to the preparation's tonicity *in vivo*, as discussed above.

Table 2. Characterization of the optimized nanoemulsions. Zero shear viscosity was always determined at 250 rpm (Newtonian behavior) except for N_{FOS} 4:6 at 32 ºC, which showed non-Newtonian behavior, and was therefore assessed by measuring at several rotational speeds and inferred from linear regression analysis. Mean size, PDI and zeta potential are presented for formulation that underwent 5 extrusion cycles (N_{FOS} 4:6), or 9 extrusion cycles (N_{FOS} 1:9).

Formulation		Osmolality (mOsmol/kg)	Mean size (nm)	PDI	Zeta potential (mV)	Viscosity at 20 ºC (cP)	Viscosity at 32 ºC (cP)
N_{FOS} 1:9	Mean	695	216.4	0.305	-20.8	2.15	1.56
	SD	2	10.5	0.031	3.9	-	-
	RMPS	3	6	6	9	1	1
	n	1	1	1	1	1	1
N_{FOS} 4:6	Mean	1611	209.2	0.263	-18.6	22.73	20.05
	SD	7	21.7	0.036	0.5	-	0.098
	R^2	-	-	-	-	-	0.9753
	RMPS	3	2	2	2	1	1
	n	3	2	2	1	1	1

FOS – fosphenytoin; n - number of independent formulations characterized; PDI – polydispersity index; R^2 – coefficient of determination; RMPS - replicate measurements per sample, reflected in mean and SD if n = 1; SD – standard deviation.

If the prepared emulsions were left to stand at room temperature for at least 30 minutes before size characterization, a thin layer of cream-like appearance was formed. If samples were taken from the middle of the rested preparation the droplet size was nanometric, but a little above the desired limit, and polydispersity was high, as could be expected from spontaneous emulsification (data not shown). If the preparations were energetically stirred before size determination, the polydispersity would be too high for size characterization, due to the redispersion of the components that, at rest, accumulated at the surface. To increase size homogeneity, premix membrane homogenization was tested, and the results are shown in Figure 3. This time, characterization was done immediately after homogenization.

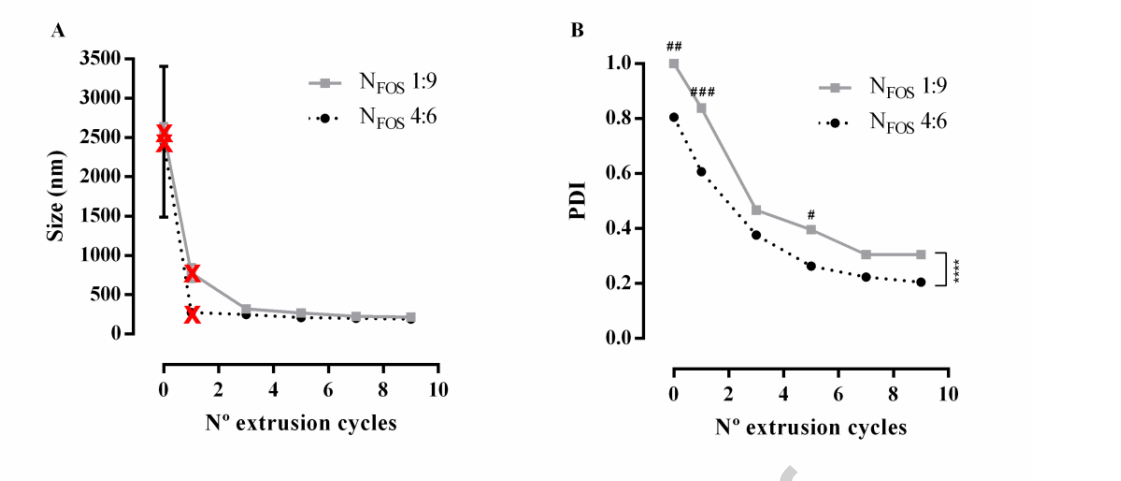

Figure 3. Premix membrane emulsification effect on droplet size dispersion of the N_{FOS} 1:9 and N_{FOS} 4:6 emulsions. Mean size (A) and PDI (B) dependence on the increasing number of extrusion cycles. Data are shown as mean ± SEM. Red cross mark signals mean size results that showed poor quality, consequently being unreliable. **** $p < 0.0001$ for formulation factor in two-way ANOVA analysis; $^{\#}$ $p < 0.05$; $^{\#\#}$ $p < 0.01$; $^{\#\#\#}$ $p < 0.001$ in Sidak's multiple comparisons post-test; FOS – fosphenytoin; N – nanoemulsion; PDI - polydispersity index; SEM - standard error of the mean.

Droplets' mean size was not significantly different between formulations, but PDI was ($p < 0.0001$), with the post-test showing significance up to the 5th extrusion cycle. Overall, the increasing number of extrusion cycles significantly reduced both mean size and PDI ($p < 0.0001$) for both formulations, but there was no further significance from the 3rd extrusion cycle on. Thus, 5 extrusion cycles were selected for the homogenization of N_{FOS} 4:6. Nevertheless, while the statistical analysis and conclusion was similar for N_{FOS} 1:9, size (266 ± 40 nm) and PDI (0.396 ± 0.111) remained above the desired limit (around 200 nm for size, and below 0.3 for PDI). Therefore, for this formulation we decided to choose 9 extrusion cycles. A summary of the selected nanoemulsions' characterization parameters is shown in Table 2.

At this stage, while drug strength was successfully increased, viscosity values were low (Table 2). This might be adequate for nasal administration in the spray form, or for fast drug release. However, higher viscosity might slow down drug release and promote the retention of the preparation in the nasal cavity for longer periods, by reducing mucociliary clearance (Pires and Santos, 2018). Hence, for extended drug release we sought to increase the preparation's viscosity.

4.2 Pluronic and Carbopol thermosensitive emulgel development

From the former leading preconcentrate, a gelling emulsion (thermosensitive emulgel) was developed, based on previously reported Pluronic/Carbopol thermosensitive gels (Serralheiro et al., 2014). In contrast to the previous work by Serralheiro *et al.* (2014), we chose to express the concentration in w/w percentage instead of w/v because it increases precision in preparation, and

took care in neutralizing Carbopol and controlling all the preparations' final pH, assuring that all were close to neutrality, where Carbopol has maximum viscosity (data not shown). After screening different Pluronic concentrations, the aqueous phase was set at 90%, composed of 17% Pluronic (w/w), 0.2% Carbopol (w/w) and 22.3 mg/g of fosphenytoin. The reduction of fosphenytoin's strength was necessary to not increase the osmolality of thermosensitive gel too much (TG_{PC+FOS}, Table 3). This resulted in a thermosensitive emulgel (TE_{PC+FOS}) with a drug strength equivalent to 15 mg/g of phenytoin (1.5%) and an osmolality about 2-fold higher than that of the thermosensitive gel (Table 3). This is due to the presence of the cosolvent, as explained before, but the osmolality of the aqueous phase before emulsification was within safe limits.

Table 3. Osmolality, mean size (when applicable) and rheological characterization of the thermosensitive gels and emulgel. For Newtonian fluids zero shear viscosity was considered to be the value matching the highest torque; for non-Newtonian fluids it was inferred from regression analysis, as described in section 2.5.

		Osmolality (mOsmol/kg)	Mean size (nm)	PDI	Zero shear viscosity at 15 °C (cP)	T_{gel}50 (°C)	T_aMax (°C)
TG_{PC}	Mean	n.d.	n.d.	n.d.	1663.4	#	#
	SD				- 14.3; + 14.4		
	n				4		
	R^2				0.9998		
$1TG_{PC+FOS}$	Mean	626	n.d.	n.d.	32.0	25.3	24.2
	SD	2			1.70	0.3	
	n	3			4	4	
	RMPS	3			1	1	
	R^2	-			-	0.9265	
TE_{PC+FOS}	Mean	1405	358.3	0.277	184.3	25.9	24.4
	SD	32	91.5	0.156	16.23	0.6	
	n	7	7	7	7	7	
	RMPS	3	2	2	1	1	
	R^2	-	-	-	-	0.9167	

FOS – fosphenytoin; n - number of independent formulations characterized; n.d. - not determined; PC – Pluronic and Carbopol; PDI – polydispersity index; R^2 – coefficient of determination; RMPS - replicate measurements per sample, reflected in mean and SD if n = 1; SD - standard deviation; T_aMax – maximum

acceleration temperature; TE – thermosensitive emulgel; TG – thermosensitive gel; $T_{gel}50$ – half-gelation temperature; # - not possible to determine, ambiguous fit.

Despite the presence of polymers in the aqueous phase, an emulsion of nanometric size was still obtained (Table 3). However, the mean droplet size was quite higher than 200 nm, the limit considered by some authors for nanoemulsions, even if likely overestimated by using a dilution of only 25-fold, due to the approximation of considering the viscosity of the diluted sample that of water (Pires and Santos, 2018).

The effects of the presence of the fosphenytoin salt and of emulsification in the rheological behavior of the Pluronic/Carbopol dispersion were evaluated by comparing the thermosensitive gel with or without the drug and the resulting thermosensitive emulgel. There was a substantial effect of both fosphenytoin and emulsification on the apparent viscosity of the Pluronic/Carbopol sol (before transition to gel) (Figure S1A, supplementary data). Fosphenytoin caused a dramatic decrease in the viscosity of the thermosensitive gel at lower temperatures, probably because it interferes with Carbopol, which is known to be incompatible with high electrolyte levels (Rowe et al., 2009). The dispersion of the preconcentrate in the gel increased the viscosity again, partially compensating the previous effect, but pseudoplasticity, likely an effect of Carbopol in the original thermosensitive formulation, was lost.

In the present work, we chose to determine and report zero shear viscosity (Table 3), given its relevance for the physical stability of the formulations and for drug diffusion after administration, and because reporting this parameter may reduce the difficulty of comparison between formulations, and between studies, due to speed and spindle variation. In the case of Newtonian fluids, zero shear viscosity is the characteristic viscosity of the preparation (same for all shear rates), but for non-Newtonian fluids it was inferred from regression analysis, as described in section 2.5.

In contrast to the viscosity of the sol state, the gelation temperatures were not significantly different ($p > 0.05$, F test) (Figure S1B, supplementary data). As the sol-gel transition did not occur abruptly at a single temperature value, we chose to determine the temperature at the middle of the steep transition in viscosity, and called it half-gelation temperature ($T_{gel}50$). To do so, and trying to minimize subjectivity as much as possible, a non-linear regression model was used whenever applicable, as described in section 2.5. The estimated half-gelation temperatures of TG_{PC+FOS} and TE_{PC+FOS} were between 25 and 26 °C (Table 3). As the viscosity of the gel sometimes increased over the measurable limit of the viscometer apparatus at the applied speed, an uncertainty on the maximum viscosity was created and, therefore, the estimation of the half-gelation temperature became ambiguous. In such cases, the point of maximum acceleration in the increase of viscosity could be a more precise comparison parameter of sol-gel transitions between formulations, although it does not allow to test for statistical differences. Maximum acceleration temperatures were also very similar in TG_{PC+FOS} and TE_{PC+FOS} (Table 3).

Although, at this stage, we were able to produce relatively stable thermosensitive emulgels, with a reasonable amount of drug, their gelation temperature was too low for nasal instillation, especially

during the summer, requiring them to be refrigerated immediately prior to a possible administration, in order to have a viscosity low enough to allow handling. Therefore, we decided to try to increase the formulations' gelation temperature. In contrast, if topical cutaneous application is intended, the complete gelation occurring between 25 - 30 ºC might be beneficial, ensuring that a complete semisolid consistency is obtained shortly after spreading on the skin.

4.3 Pluronic only thermosensitive nanoemulgel development

In order to produce formulations with a more desirable viscosity at room temperature, for intranasal instillation purposes, we decided to decrease Pluronic concentration to 16% (w/w) and eliminate the Carbopol from the aqueous phase composition, while using the same preconcentrate as before, producing formulations with a 40% (TN_{P+FOS} 4:6) or 10% (TN_{P+FOS} 1:9) (w/w) preconcentrate proportion. The preconcentrate contained 10 mg/g of phenytoin and the aqueous phase 25 mg/g of fosphenytoin, thus the drug strength for the two studied proportions corresponded to 14.4 and 16.7 mg/g of phenytoin equivalents, respectively.

Both resulting emulsions were white and opaque, but while formula TN_{P+FOS} 1:9 gave rise to an apparently homogeneous formulation, TN_{P+FOS} 4:6 contained a few small transparent aggregates. These aggregates appeared to be amorphous when viewed under an optical microscope, and we suppose they were Pluronic coacervates. In contrast, in TN_{P+FOS} 1:9 some cream formation occurred when the formulation was stored at 4 ºC overnight. Nevertheless, it was able to return to being homogeneous with mild agitation.

Both formulations originated dispersions with nanometric but relatively high droplet mean size and PDI values (Figure 4), suggesting heterogeneity, therefore we decided once more to perform premix membrane homogenization. Mean size and PDI were significantly different between formulations ($p < 0.0001$), but the post-test only had significance up to the 3rd extrusion cycle. The increasing number of extrusion cycles also significantly reduced both mean size and PDI ($p < 0.0001$), in general, but there was no further significant reduction on either size or PDI from the 3rd extrusion cycle on. Thus, 5 extrusion cycles were selected for the homogenization of these nanoemulsions.

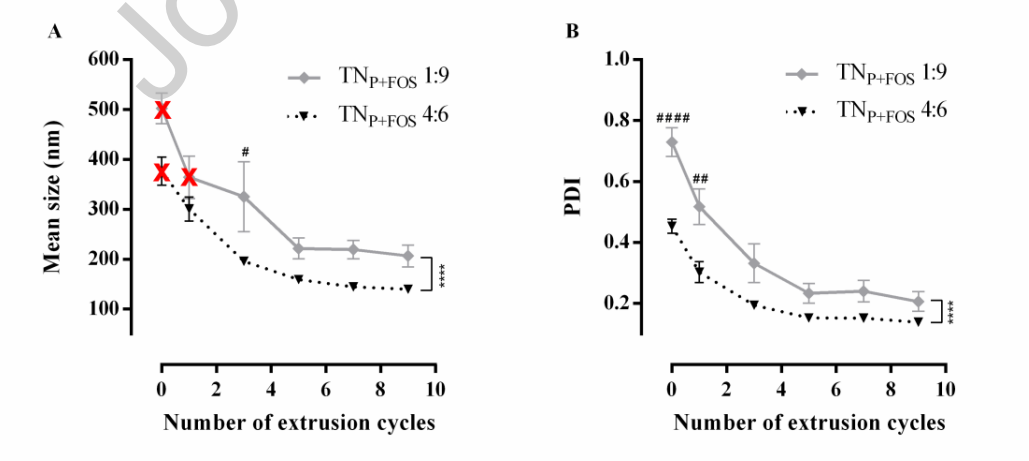

Figure 4. Premix membrane emulsification effect on droplet size dispersion of the TN_{P+FOS} 4:6 and TN_{P+FOS} 1:9 nanoemulgels. Mean size (A) and PDI (B) dependence on the increasing number of extrusion cycles. Data are shown as mean ± SEM. Red cross mark signals mean size results that showed poor quality, consequently being unreliable. **** $p < 0.0001$ for formulation factor in two-way ANOVA analysis; $^{\#}$ $p < 0.05$; $^{\#\#}$ $p < 0.01$; $^{\#\#\#\#}$ $p < 0.0001$ in Sidak's multiple comparisons post-test; FOS – fosphenytoin; P – Pluronic; PDI - polydispersity index; SEM - standard error of the mean; TE – thermosensitive emulgel.

Noticeably, after extrusion the Pluronic aggregates disappeared from the nanoemulsion TN_{P+FOS} 4:6, and did not form again. As for TN_{P+FOS} 1:9, cream formation continued to occur with storage at 4 ºC, but agitation still returned the formulations to being homogeneous, as before. Furthermore, formulations mean size and PDI did not increase over at least 2 weeks at 4 ºC, and zeta potential was only slightly negative, in practical terms basically neutral, as expected, since all the excipients that were used were neutral (Figure S2, supplementary data).

Both nanoemulgels' rheological behavior was evaluated. The nanoemulgel TN_{P+FOS} 4:6 exhibited a pseudoplastic non-Newtonian behavior at all studied temperatures. In comparison to TN_{P+FOS} 1:9, it was more viscous at both 15 and 20 ºC, but much less viscous at 32 ºC (Figure S3A, supplementary data), since it did not undergo sol-gel transition at all in the studied temperature range (20 to 42 ºC, Figure S3B, supplementary data). This might be due to the high proportion of Transcutol mixing with the aqueous phase, resulting in Pluronic dilution below the minimum concentration required for gelation. As for TN_{P+FOS} 1:9, the gelation was more variable and with a slightly but significantly higher $T_{gel}50$ ($p < 0.0001$) in comparison with the respective TG_{P+FOS} (Figure S3B, supplementary data), which indicated that emulsification delayed the gelation, in relation to the thermogel alone. Likewise, the T_aMax of the nanoemulgel was higher than the respective thermogel (Table 4), with the nanoemulgel therefore initiating the gelation process later, with a T_aMax of about 30 ºC, high enough to prevent gelation from occurring at most common room temperatures, and still ensuring that it will occur at nasal temperature. Even if gelation is not complete at nasal temperature, the zero shear viscosity of this formulation at 32 ºC (Table 4) is already more than enough to be expected to promote retention in the nasal cavity and, consequently, sustained release. Other formulation characterization parameters of the final chosen thermosensitive nanoemulgel TN_{P+FOS} 1:9 are also shown in Table 4, as well as the remaining characterization parameters for TG_{P+FOS} and TN_{P+FOS} 4:6.

Table 4. Osmolality, size, zeta potential and rheological characterization of the thermosensitive gel and nanoemulgels. For Newtonian fluids zero shear viscosity was assessed by measuring at several rotational speeds and considered to be the value matching the highest torque; for non-Newtonian fluids it was inferred from regression analysis (with prior logarithmic transformation for 32 ºC) as described in section 2.5.

		Osmolality (mOsmol/kg)	**Mean size (nm)**	**PDI**	**Zeta potential (mV)**	**Zero shear viscosity (cP)**		$T_{gel}50$	T_aMax
						At 20 ºC	At 32 ºC		
TG_{P+FOS}	**Mean**	600	n.d.	n.d.	n.d.	57.4	116145	29.5	27.6
	SD	14				1.6	- 1810; + 1838	0.11	

	n	5				3	3	3	
	RMPS	3				1	1	1	
	R^2	-				-	0.9995	0.9985	
TN_{P+FOS} 1:9	**Mean**	1375	219.7	0.237	-10.7	233.3	168655	33.7	30.3
	SD	52	26.8	0.040	2.7	0.4	- 11492; + 12333	0.15	
	n	4	7	7	4	3	5	3	
	RMPS	3	2 to 3	2 to 3	2 to 3	1	1	1	
	R^2	-	-	-	-	0.9943	0.9858	0.9990	
TN_{P+FOS} 4:6	**Mean**	#	159.8	0.154	n.d.	407.5	352.0	#	#
	SD		6.0	0.018		0.9	2.3		
	n		3	3		1	1		
	RMPS		2 to 4	2 to 4		1	1		
	R^2		-	-		0.9575	0.9443		

FOS – fosphenytoin; n – number of independent formulations characterized; n.d. - not determined; P – Pluronic; PDI – polydispersity index; R^2 – coefficient of determination; RMPS - replicate measurements per sample, reflected in mean and SD if n = 1; SD – standard deviation; T_aMax – maximum acceleration temperature; T_{gel}50 – half-gelation temperature; TG – thermosensitive gel; TN – thermosensitive nanoemulgel; # – not possible to determine, no freezing or no gelling occurred.

4.4 HPLC method validation

The validated HPLC method was based on the one developed by Antunes Viegas et al. (2016). Chromatographic conditions were kept the same, but sample processing was simplified, since the matrix was only buffer plus cosolvent, with no need for the addition of acid. In this case, sample processing consisted of dilution only, in order to obtain drug levels within the range of the calibration curves.

The analysis of blank samples [nasal simulant buffer plus Transcutol at 30% (w/w)] confirmed the absence of substantial interferences at the retention times of the analytes of interest (no peak at all, or peak with an area of less than 20% of that of the LLOQ). The same occurred for the formulation vehicles, which also had no interferences at those retention times. Figure S4 (supplementary data) shows a chromatogram for one of the calibration standards, demonstrating typical mean retention times: approximately 4 minutes for fosphenytoin and 10 minutes for phenytoin.

Calibration curves' range was defined as 0.1 - 40.0 μg/mL for both fosphenytoin and phenytoin. Linearity was observed for both analytes' mean curves (R^2 = 0.9976 for fosphenytoin and R^2 = 0.9980 for phenytoin) (Table S1, supplementary data). The LLOQ's were experimentally determined and set at 0.1 μg/mL, with adequate precision and accuracy, for both fosphenytoin and phenytoin (|bias| and |CV| < 8%). QC samples also showed precision and accuracy within the acceptance criteria in intra and interday evaluations (|bias| and |CV| < 5%) (Table S2, supplementary data). Absolute recovery, determined for 3 concentration levels (QC_1, QC_2 and QC_3), was between 93 and 101% for fosphenytoin, and 95 and 100% for phenytoin. All values had an associated CV of less than 5% (Table S3, supplementary data). Analyte stability was also evaluated by submitting samples, at two concentration levels (QC_1 and QC_3), to different time and temperature conditions, in order to better predict possible degradation during handling and/or storage. Both analytes proved to be stable while being kept at room temperature for 24 hours, and also when submitted to 3 cycles of freeze/thaw, in 3 consecutive days, showing no substantial degradation (Table S4, supplementary data).

Validation was successfully completed, with all evaluated parameters fitting the acceptance criteria. Furthermore, samples were stable under relevant conditions, which gives further confidence in the method's usefulness and reliability.

4.5 Drug release study

For the selected nanoemulsions (N_{FOS} 1:9 and N_{FOS} 4:6) and thermosensitive nanoemulgel (TN_{P+FOS} 1:9) fosphenytoin and phenytoin release profiles were evaluated. For comparison purposes, we also evaluated the release rate of an aqueous drug solution having fosphenytoin at 1.3 mg/g, phenytoin at 0.2 mg/g and Transcutol at 30% w/w (FPT solution).

Percentual drug release and percentual drug release rates are shown in Figure 5 and Table 5. We expected to have an overall faster, immediate release of fosphenytoin from the nanoemulsions and a slower, sustained release from the thermosensitive nanoemulgel, due to the much greater zero shear viscosity at 32 ºC of the nanoemulgel, which was expected to reduce drug diffusion. Indeed, the percentual release of fosphenytoin from N_{FOS} 1:9 was the fastest ($p < 0.0001$ in relation to all other formulations) and its nanoemulgel counterpart, TN_{P+FOS} 1:9, led to a slower release ($p < 0.0001$). But the initial hypothesis was not verified for N_{FOS} 4:6, since even while having a much lower zero shear viscosity than TN_{P+FOS} 1:9, it was slower at releasing fosphenytoin ($p < 0.0001$). Moreover, TN_{P+FOS} 1:9 also had a higher fosphenytoin percentual release than the FPT solution, which given its simple composition and very low viscosity was expected to have the most effective release. These results might have been due to the higher Transcutol percentage that exists in the FPT solution (30% w/w) and also in N_{FOS} 4:6 (13.6% w/w), since N_{FOS} 1:9 had less cosolvent (only 3.4% w/w). Another possibility was that since N_{FOS} 4:6 has a higher oil phase proportion it could adsorb fosphenytoin, or since it also has a higher amount of phenytoin it could also be interacting with its prodrug and slowing its release.

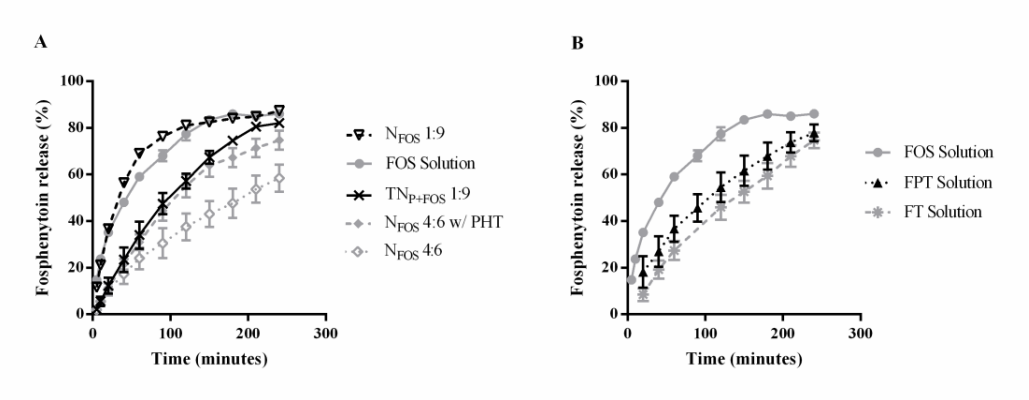

Figure 5. Fosphenytoin's percentual drug release between 5 and 240 minutes, for nanoformulations (A) and solutions (B). The aqueous solution containing fosphenytoin only (FOS solution) appears in both graphics, since it is the most reliable positive control. FOS – fosphenytoin; FPT – fosphenytoin, phenytoin and Transcutol; FT – fosphenytoin and Transcutol; N – nanoemulsion; P – Pluronic; PHT – phenytoin; TN – thermosensitive nanoemulgel.

Table 5. Fosphenytoin's percentual drug release rate and significance matrix of the difference between formulations. Rate constant calculated by applying a linear regression to the plotting of the square root of time ($X = \sqrt{X}$) versus percentual drug release divided by the area of the membrane used in the assay ($Y = Y/0.64$). Slopes were then compared using an F test.

Drug	Formulation	R^2 (R^{2}')	Drug release rate ($\%\cdot cm^{-2}\cdot t^{1/2}$)		*Significance of differences in rate between formulations* (*p* value)					
			Mean	SD	N_{FOS} 1:9	N_{FOS} 4:6	N_{FOS} 4:6 w/ PHT	FPT Solution	FOS Solution	FT Solution
Fosphenytoin	TN_{P+FOS} 1:9	0.9809 (0.9964)	10.44	0.34	****	****	NS	NS	0.0122	NS
	N_{FOS} 1:9	0.9901 (0.9980)	16.50	0.46		****	****	****	****	****
	N_{FOS} 4:6	0.8791 (0.9982)	6.61	0.44			****	0.0210	****	0.0006
	N_{FOS} 4:6 w/ PHT	0.9477 (0.9924)	9.75	0.46				NS	0.0157	NS
	FPT Solution	0.8258	8.77	0.86					0.0306	NS

		(0.9992)						
	FOS Solution	0.9909 (0.9969)	12.33	0.33				0.0104
	FT Solution	0.9270 (0.9982)	9.23	0.55				
Phenytoin	**TN_{P+FOS} 1:9**	0.8173 (0.9770)	5.73	0.72	0.0301	NS	NS	
	N_{FOS} 1:9	0.9690 (0.9981)	7.14	0.24		****	NS	
	N_{FOS} 4:6	0.6578 (0.9972)	4.03	0.62			0.0032	
	FPT Solution	0.6909 (0.9900)	8.12	1.25				

FOS – fosphenytoin; FPT – fosphenytoin, phenytoin and Transcutol; FT – fosphenytoin and Transcutol; N – nanoemulsion; NS – not significant (statistical difference); P – Pluronic; PHT – phenytoin; R^2 – linear regression's coefficient of determination, using all individual values corresponding to each individual time point; R^{2}' – linear regression's coefficient of determination, using mean values for each time point; SD – standard deviation; TN – thermosensitive nanoemulgel; w/ - without. **** $p < 0.0001$.

A fosphenytoin aqueous solution (FOS solution, with no phenytoin and no Transcutol) and a fosphenytoin plus Transcutol solution (FT solution, with no phenytoin) were also tested to better clarify the previous results. Indeed, fosphenytoin's release from the FOS solution was significantly faster ($p < 0.05$) than that of the other solutions, which seems to confirm that Transcutol at a higher percentage did in fact inhibit the release of fosphenytoin. The presence or absence of phenytoin did not seem to affect the release of the prodrug, since the FPT and FT solutions showed no significant differences in percentual drug release rate and their release profiles were very similar. Additionally, we also evaluated the drug release from a formulation equivalent to N_{FOS} 4:6, but without phenytoin (N_{FOS} 4:6 w/ PHT). The results imply that, in this case, the presence of phenytoin does decrease the release of fosphenytoin from the nanoformulations, since in N_{FOS} 4:6 w/ PHT the prodrug's percentual release was faster than in N_{FOS} 4:6 ($p < 0.0001$). A possible explanation for this could be an attachment of fosphenytoin to the surface of the oil droplets containing phenytoin, therefore delaying/inhibiting its release.

As for the percentual release of phenytoin from the formulations (Figure 6 and Table 6), most results were as expected, since FPT solution was the fastest, followed by N_{FOS} 1:9, and with N_{FOS} 4:6 and TN_{P+FOS} 1:9 coming last. Nevertheless, TN_{P+FOS} 1:9 practically matched the release profile

of N_{FOS} 4:6. Again, it might be the high amount of Transcutol present in N_{FOS} 4:6 that could decrease drug release. Moreover, given its very low solubility, we were only able to solubilize, and therefore quantify, low amounts of phenytoin, and consequently the linear fit for the data regarding this drug was less good than for fosphenytoin, since the error associated with the quantification and variability between chambers is more substantial. Nevertheless, when considering the mean values for each time point (instead of using all individual values), the linear fit improves substantially, which seems to further confirm the influence of the variability between chambers.

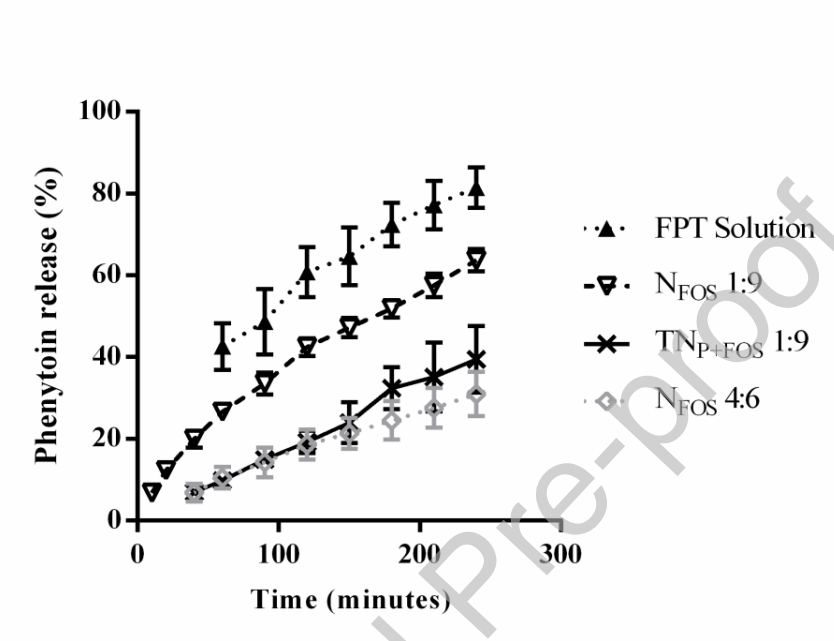

Figure 6. Phenytoin percentual release between 5 and 240 minutes. FOS – fosphenytoin; FPT – fosphenytoin, phenytoin and Transcutol; N – nanoemulsion; P – Pluronic; TN – thermosensitive nanoemulgel.

When comparing the percentual drug release of both drugs in the nanoformulations, phenytoin was always released in a lesser extent than fosphenytoin. This might have been due to the fact that while fosphenytoin is in the external phase, solubilized and free to pass through the membrane, phenytoin is most likely to be located inside the internal phase's droplets. Hence, it will either have to be released from the droplets to pass the membrane, or be transported inside the droplets themselves, and although droplet size may be around 200 nm, a considerable amount of droplets might not pass the membrane.

In what concerns cumulative drug quantity (Figure 7), and comparing the most promising formulations only (no controls), N_{FOS} 1:9 had the highest fosphenytoin release, which was expected since it had the highest content of this drug. N_{FOS} 4:6 had the highest phenytoin release, since the greater oil to water proportion made it the formulation with its highest amount. Therefore, if the purpose is to choose the formulation with the highest amount of phenytoin in the active form as possible, N_{FOS} 4:6 is ideal. Nevertheless, if considering phenytoin equivalents, N_{FOS} 1:9 has a much higher content than any of the other formulations, and should, therefore, be selected.

As for the cumulative drug quantity release rate (Table 6), the fastest formulations were the ones that had a higher amount of each drug: N_{FOS} 1:9 had the fastest fosphenytoin release, followed by TN_{P+FOS} 1:9 and N_{FOS} 4:6; and N_{FOS} 4:6 had the fastest phenytoin release, followed by N_{FOS} 1:9 and TN_{P+FOS} 1:9.

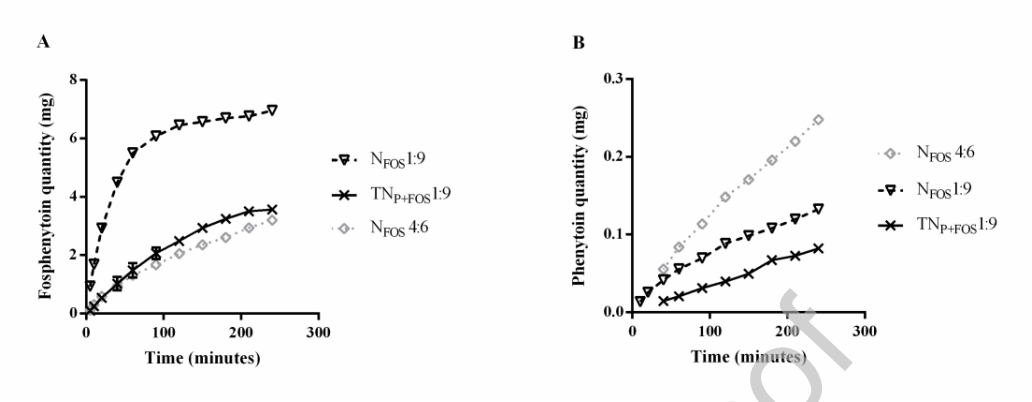

Figure 7. Cumulative drug quantity release for the most relevant formulations, for fosphenytoin (A) and phenytoin (B). FOS – fosphenytoin; N – nanoemulsion; P – Pluronic; TN – thermosensitive nanoemulgel.

Table 6 – Cumulative drug quantity release rate and significance matrix of the difference between formulations. Release rate was calculated by applying a linear regression to the plotting of the square root of time ($X = \sqrt{X}$) versus cumulative drug quantity divided by the area of the membrane used in the assay ($Y = Y/0.64$). Slopes were then compared using an F test.

Drug	Formulation	R^2 ($R^{2'}$)	Drug release rate ($mg \cdot cm^{-2} \cdot t^{-1/2}$)		Significance of differences in rate between formulations (*p* value)	
			Mean	SD	N_{FOS} 1:9	N_{FOS} 4:6
Fosphenytoin	**TN_{P+FOS} 1:9**	0.9809 (0.9964)	0.454	0.015	****	0.0099
	N_{FOS} 1:9	0.9901 (0.9980)	1.315	0.037		****
	N_{FOS} 4:6	0.8791 (0.9982)	0.362	0.024		
Phenytoin	**TN_{P+FOS} 1:9**	0.8135 (0.9758)	0.012	0.002	0.0260	0.0024
	N_{FOS} 1:9	0.9693	0.015	0.001		****

	(0.9981)		
N_{FOS} 4:6	0.6575	0.032	0.005
	(0.9970)		

FOS – fosphenytoin; FPT – fosphenytoin, phenytoin and Transcutol; FT – fosphenytoin and Transcutol; N – nanoemulsion; P – Pluronic; PHT – phenytoin; R^2 – linear regression's coefficient of determination, using all individual values corresponding to each individual time point; $R^{2'}$ – linear regression's coefficient of determination, using mean values for each time point; SD – standard deviation; TN – thermosensitive nanoemulgel. **** $p < 0.0001$.

Given the results, and considering not only release profiles, but also drug strength, we conclude that from the developed formulations: 1) N_{FOS} 1:9 (27.0 mg/g of phenytoin equivalents) could be useful for fast release, having potential for the treatment of *status epilepticus* or acute pain episodes, where a fast onset is needed; and 2) TN_{P+FOS} 1:9 (16.7 mg/g of phenytoin equivalents) and N_{FOS} 4:6 (22.0 mg/g of phenytoin equivalents), having a more sustained release, could be possibly beneficial for chronic epilepsy or nasal wound healing.

5. Conclusion

Phenytoin is a drug with poor water solubility, not solubilizing well in lipids either, but having a substantially higher solubility in Transcutol. In this work, it is shown that Transcutol is important in order to have phenytoin in the soluble form, even in emulsions, but it is not enough to promote the desired drug strengths when aiming for systemic therapeutic effects.

The formulation strategy selected for this work implicated a simple procedure of spontaneous emulsification, followed by premix membrane homogenization to reduced droplet size heterogeneity and mean size. It resulted in the successful development of two aqueous liquid nanoemulsions, composed of Miglyol 812, Tween 80, Transcutol, phenytoin, and fosphenytoin, (N_{FOS} 1:9 and N_{FOS} 4:6) and one thermosensitive nanoemulgel, with the same composition plus Pluronic in the external phase (TN_{P+FOS} 1:9), all potentially suitable for intranasal administration. The association of the soluble prodrug, fosphenytoin, with the parent drug, phenytoin, increased drug strength to the equivalent of 22 mg/g or 27 mg/g of phenytoin in the lead nanoemulsions, and 16.7 mg/g in the lead nanoemulgel, which could be considered reasonably high for this drug. The association of fosphenytoin interfered, however, with formulation's characteristics, as it leads to a steep increase in osmolality, since it is a disodium salt, limiting its concentration for safety reasons. Fosphenytoin also reduced Carbopol's viscosification properties, but not Pluronic's gelation temperature.

The selected low viscosity nanoemulsions had an immediate or prolonged release profile, depending of anhydrous phase proportion: for 10% (N_{FOS} 1:9) the formulation had an immediate release profile; for 40% (N_{FOS} 4:6) the preparation had a prolonged release. The thermosensitive nanoemulgel (TN_{P+FOS} 1:9) showed prolonged drug release, as expected. Whether these formulations are more suited for topical effects or therapeutic brain delivery is an issue that should be addressed in future studies.

6. Acknowledgements and funding

We thank JPN Pharma (Tarapur, India) for donating fosphenytoin sodium. Patrícia C. Pires and part of the work was supported by European Regional Development funds through the Operational Programme "Centro 2020", through the ICON project (Interdisciplinary Challenges On Neurodegeneration, reference CENTRO-01-0145-FEDER-000013). Part of the work was supported by national funds through "Fundação para a Ciência e a Tecnologia" (reference UID / Multi / 00709/2019).

7. Declarations of interests

The authors declare no conflicts of interest.

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Graphical abstract