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The promise of prodrugs and nanosystems in nose-to-brain delivery of poorly soluble drugs: the case of phenytoin and fosphenytoin

Patrícia Sofia Cabral Pires

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Supervisor: Adriana Oliveira dos Santos, PhD
Co-supervisor: Gilberto Lourenço Alves, PhD
Co-supervisor: Márcio José de Abreu Marques Rodrigues, PhD

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Professora Doutora Ana Paula Coelho Duarte, Professora Catedrática da Universidade da Beira Interior

Professor Doutor António José Leitão das Neves Almeida, Professor Catedrático da Faculdade de Farmácia da Universidade de Lisboa

João Nuno Sereno de Almeida Moreira, Professor Auxiliar da Faculdade de Farmácia da Universidade de Coimbra

Professora Doutora Adriana Oliveira dos Santos, Professora Auxiliar da Universidade da Beira Interior

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Preface

The work presented in this thesis aimed to find a solution to a problem associated with many drugs: low aqueous solubility, especially important in the case of intranasal delivery, where the administration volume is low, which poses an additional challenge. This characteristic makes these molecules hard to formulate as solutions without the use of a high amount of organic solvents, which are potentially toxic. We hypothesized that a water-soluble prodrug that would be readily converted into its parent drug prior or after absorption, even if less permeble, could be a safe and effective alternative of increasing these type of drugs' solubility without having to use potentially harmful excipients. Hence, a drug/prodrug pair already existing in the market – phenytoin, a low solubility antiepileptic drug, and fosphenytoin, its phosphate ester prodrug – were selected. The main objective of this work was, therefore, to serve as a proof of concept that phosphate ester prodrugs can be a useful strategy in nasal formulation development to safely increase drug strenght of low solubility drugs, consequently increasing their bioavailability.

This thesis is divided into five chapters plus a concise section of overall concluding remarks. Most of the data has been previously published – either in international scientific indexed journals or as a book chapter. Only Chapter 5 was still under preparation for submission at the time this thesis was written.

Chapter 1 is an introductory contextualization, addressing topics such as epilepsy (pathophysiology and treatment) and intranasal administration (how it works, advantages and disadvantages, intranasal delivery of antiepileptics), providing a theoretical basis for the experimental work. Most of the information presented in this chapter was published in the book chapter "*Intranasal delivery of antiseizure drugs*, Drug Delivery Devices and Therapeutic Systems, 2020 (1st ed.), Elsevier Academic Press (ISBN: 9780128198384)".

Chapter 2 is a systematic review of the scientific literature regarding non-clinical studies that assessed the intranasal delivery of small molecule drugs within nanosystems. The purpose of this review was to better understand the variables that can influence the outcomes of *in vivo* studies comprising intranasal administration, as well as to assess whether there is a specific nanosystem type that is superior at achieving high brain targeting through this route. The data presented in this chapter corresponds to the article

"*Nanosystems in nose-to-brain drug delivery: A review of non-clinical brain targeting studies*, Journal of Controlled Release, 2018 (DOI: 10.1016/j.jconrel.2017.11.047)".

Chapter 3 reports the experimental work regarding the development, *in vitro* characterization and *in vivo* pharmacokinetic study of simple fosphenytoin formulations. The purpose of this work was to assess whether simple formulations containing this prodrug could already achieve high brain drug levels despite their simplicity, and to determine whether fosphenytoin could reach the brain in an unmetabolized form or if it was completely converted to phenytoin by the time it got there. This work was published in the article "*Intranasal fosphenytoin: the promise of phosphate esters in nose-to-brain delivery of poorly soluble drugs*, International Journal of Pharmaceutics, 2020 (DOI: 10.1016/j.ijpharm.2020.120040)".

Chapters 4 and 5 are about the development of nano or microemulsions containing phenytoin and fosphenytoin, including *in vitro* and *in vivo* characterization. It was an effort to produce formulations also containing the drug in the active and diffusible form (phenytoin), in addition to the prodrug (fosphenytoin), to assess whether it could make brain targeting faster. Chapter 4 was published in the article "*Nanoemulsions and thermosensitive nanoemulgels of phenytoin and fosphenytoin for intranasal administration: formulation development and in vitro characterization*, European Journal of Pharmaceutical Sciences, 2020 (DOI: 10.1016/j.ejps.2019.105099)", and Chapter 5 was being prepared for submition at the time this thesis was written.

When including the published works into the thesis, some alterations were made in order to increase coherence and uniformity throughout the text, for example by decreasing the number of abbreviations or making them homogeneous, or by summarizing the "Introduction" sections of the articles to give origin to the brief introductory sections of Chapters 2 to 5.

Finaly, after Chapter 5, a concise section was added as a final discussion of the most important aspects of the work, and providing a conclusion for the thesis as a whole.

Abstract

In brain-targeted drug delivery, the intranasal route can be a good alternative to parenteral administration. In addition to being associated with a greater comfort for the patient, intranasal drug delivery can reduce systemic drug distribution, resulting in an increased safety, and can allow direct drug transport to the brain, resulting in an increased therapeutic efficacy. For example, benzodiazepines administration for the treatment of acute epileptic episodes has been proven to be at least as effective as their intravenous administration. Nevertheless, their solubilization requires substantial amounts of organic solvents, which can cause lacrimation and nose and throat irritation. Additionally, benzodiazepines can cause somnolence, deleterious cognitive effects and dependence/tolerance.

Phenytoin is also an antiepileptic drug, being non-inferior in efficacy, while not having these adverse effects. Although its systemic administration can cause other adverse events (such as cardiovascular complications or liver toxicity), its intranasal administration could increase its safety and even efficacy compared to other non-invasive routes in the treatment of *status epilepticus*. Yet, phenytoin has low aqueous solubility, being difficult to formulate at a high strength. However, its hydrophilic prodrug, fosphenytoin, has high water solubility. Hence, aqueous liquid water-based formulations of fosphenytoin for intranasal administration were developed. Pharmacokinetic results in mice showed that a fosphenytoin formulation containing hydroxypropyl methylcellulose and albumin prolonged drug concentration in the brain, also producing a high absolute drug bioavailability. The study demonstrated that phosphate ester prodrugs (such as fosphenytoin) can be an efficient strategy to increase the intranasal bioavailability of low solubility drugs (such as phenytoin).

In addition, we hypothesized that if there was phenytoin in the formulation, in the active and diffusible form, brain drug delivery could be increased and/or made faster. Thus, nano and microemulsions containing phenytoin (internalized in the oil droplets) and fosphenytoin (solubilized in the aqueous phase) in combination were developed. A microemulsion having good characteristics (reasonably homogeneous, with small droplet size and physically stable for at least 1 week) was selected for pharmacokinetic evaluation in mice. In addition to the intranasal administration of this selected microemulsion, containing both phenytoin and fosphenytoin, a second microemulsion was also administered intranasally, having an identical composition but without

phenytoin (with fosphenytoin only), for comparison purposes. Despite the existence of a small amount of phenytoin in the formulation not inducing accelerated brain drug delivery, it led to prolonged and increased drug levels. Moreover, the intranasal administration of the microemulsion containing both drugs led to a maximum brain concentration that was similar to that obtained with the intravenous fosphenytoin solution, also leading to prolonged drug retention. The microemulsion containing both drugs also had a higher bioavailability than any of the intranasally administered formulations containing fosphenytoin only (microemulsion without phenytoin, and simple fosphenytoin formulations). Furthermore, both microemulsions (the one containing both drugs and the one containing fosphenytoin only) led to higher drug concentrations at initial time points than those obtained with the simple intranasal fosphenytoin solution, which suggests that the microemulsion had a drug permeation enhancement effect.

Thus, in general this work allowed to prove that the use of phosphate ester prodrugs can be an effective strategy in increasing the intranasal bioavailability of low solubility drugs, albumin is a good strategy to prolong brain targeting, the existence of a small amount of active drug (in addition to the prodrug), in an emulsified form, can increase drug levels at longer time points, and the use of microemulsions can increase brain drug delivery at shorter time points.

Keywords

Albumin; Antiepileptic; Brain targeting; Fosphenytoin; Hydroxypropyl methylcellulose; Intranasal; Microemulsion; Nanoemulsion; Pharmacokinetic; Phenytoin; Prodrug; *Status epilepticus*.

Resumo

Na entrega de fármacos ao cérebro a via intranasal pode ser uma boa alternativa à administração parentérica. Para além de estar associada a um maior conforto para o doente, a via intranasal pode também reduzir a distribuição sistémica de fármaco, resultando num aumento da segurança, e possibilitar o transporte direto do mesmo para o cérebro, resultando num aumento da eficácia terapêutica. A administração de benzodiazepinas para o controlo de crises epiléticas foi já comprovada como sendo pelo menos tão eficaz como a sua administração intravenosa. No entanto, a sua solubilização requer elevadas quantidades de solventes orgânicos, o que pode causar lacrimejo e irritação no nariz e garganta. Adicionalmente, as benzodiazepinas podem causar sonolência, distúrbios cognitivos, dependência e tolerância.

A fenitoína é também um fármaco antiepilético, não sendo inferior em eficácia e não apresentando os mesmos efeitos adversos das benzodiazepinas. A sua administração sistémica pode causar outros efeitos adversos (por exemplo a nível do sistema cardiovascular ou hepático), no entanto a sua administração intranasal poderá aumentar a sua segurança e, potencialmente, a eficácia no tratamento do *status epilepticus* comparativamente a outras vias não invasivas. No entanto a fenitoína é um fármaco com baixa solubilidade aquosa, o que faz com que seja difícil de formular em dosagem elevada. Porém, o seu pró-fármaco hidrofílico, a fosfenitoína, tem uma solubilidade aquosa elevada. Assim, foram desenvolvidas formulações líquidas aquosas de fosfenitoína para administração intranasal. Os resultados de estudos farmacocinéticos em murganhos mostraram que uma formulação de fosfenitoína contendo hidroxipropilmetilcelulose e albumina prolongou a concentração de fármaco no cérebro, originando uma elevada biodisponibilidade absoluta da fenitoína. Neste estudo foi demonstrado que os pró-fármacos ésteres de fosfato (como a fosfenitoína) podem ser uma estratégia eficaz e segura no aumento da biodisponibilidade intranasal de fármacos pouco solúveis (como a fenitoína).

Adicionalmente, colocou-se a hipótese de que a presença de fenitoína na formulação, na forma ativa e pronta a difundir através das barreiras biológicas, pudesse aumentar a rapidez com que o fármaco chegava ao cérebro, e/ou a eficácia com que o fazia. Assim, foram desenvolvidas nano e microemulsões contendo fenitoína (internalizada nas gotículas oleosas) e fosfenitoína (solubilizada na fase aquosa) em combinação. Foi selecionada uma microemulsão com boas características (razoavelmente homogénea,

com tamanho de gotícula pequeno e fisicamente estável durante pelo menos uma semana) para seguir para estudos farmacocinéticos em animais. Para além da administração intranasal da microemulsão selecionada, contendo fenitoína e fosfenitoína, foi também administrada pela via intranasal uma microemulsão de composição idêntica mas sem fenitoína (só com fosfenitoína), por forma a perceber-se se a existência de uma pequena quantidade de fenitoína na forma ativa e permeável (e não apenas fosfenitoína) é necessária para obter níveis mais elevados ou acelerar a entrega de fármaco ao cérebro. Os resultados sugeriram que, apesar da existência de uma pequena quantidade de fenitoína na formulação não ter tornado a entrega de fármaco ao cérebro mais rápida, levou a um prolongamento e aumento dos níveis de fármaco. Adicionalmente, a administração intranasal da microemulsão contendo ambos os compostos (fenitoína e fosfenitoína) levou a uma concentração máxima no cérebro semelhante àquela obtida mediante administração intravenosa de fosfenitoína, levando também a um prolongamento da retenção de fármaco. A microemulsão contendo ambos os compostos teve também uma maior eficácia que qualquer outra formulação administrada pela via intranasal contendo apenas fosfenitoína (microemulsão sem fenitoína mas com fosfenitoína, e formulações simples de fosfenitoína). Para além disso, ambas as microemulsões (contendo ambos os fármacos ou apenas fosfenitoína) deram origem a concentrações superiores às obtidas com a solução de fosfenitoína intranasal, o que sugere que os excipientes que faziam parte da composição destas formulações (nomeadamente os agentes tensioativos) tiveram um efeito de aumento da permeação de fármaco.

Assim, em geral, este trabalho permitiu provar que o uso de pró-fármacos ésteres de fosfato pode ser uma estratégia eficaz no aumento da biodisponibilidade intranasal de fármacos pouco solúveis, que a albumina é uma boa estratégia de direcionamento cerebral, que a existência de uma pequena quantidade de fármaco ativo (em adição ao pró-fármaco), em emulsão, aumenta os níveis de fármaco a tempos mais longos, e que o uso de microemulsões aumenta a concentração de fármaco no cérebro a tempos curtos.

Palavras-chave

Albumina; Antiepilético; Direcionamento cerebral; Farmacocinética; Fenitoína; Fosfenitoína; Hidroxipropilmetilcelulose; Intranasal; Microemulsão; Nanoemulsão; Pró-fármaco; *Status epilepticus*.

Resumo alargado

A administração intravenosa de fármacos pode estar associada a um atraso na obtenção da resposta terapêutica, uma vez que é uma via de administração que implica o estabelecimento de acesso intravenoso, na presença de pessoal com competência para tal, o que requer geralmente hospitalização. Isto pode ser potencialmente prejudicial para o doente, especialmente em casos de emergência. O facto de a via parentérica ser invasiva está também associado a um risco de lesão no local de administração, sendo pelo menos desconfortável e, por vezes, até doloroso para o doente.

Na entrega de fármacos ao cérebro a via intranasal pode ser uma boa alternativa à administração parentérica, estando associada a um maior conforto para o doente, e tendo ainda outras vantagens, tais como a redução da distribuição sistémica de fármaco, que pode levar a um aumento da segurança da preparação, e a possibilidade de o fármaco ser transportado diretamente para o cérebro, pelas vias neuronais, que pode aumentar a eficácia uma vez que pelo menos parte do fármaco não terá que passar pela barreira hemato-encefálica.

Há hoje em dia vários produtos nasais no mercado, utilizados tanto para efeitos locais como sistémicos, sendo fáceis de administrar pelo próprio ou por um cuidador. A administração de benzodiazepinas para o controlo de crises epiléticas foi já comprovada como sendo pelo menos tão eficaz como a administração intravenosa dos mesmos fármacos, tendo chegado recentemente ao mercado duas preparações: Nayzilam®, uma formulação contendo midazolam, e Valtoco®, uma formulação contendo diazepam. No entanto, a solubilização de benzodiazepinas requer o uso de elevadas quantidades de solventes orgânicos na formulação, e estas preparações já demonstraram causar lacrimejo e irritação no nariz e garganta. Adicionalmente, as benzodiazepinas podem causar sonolência e, em caso de toma excessiva, distúrbios cognitivos, dependência e tolerância.

Tal como as benzodiazepinas, a fenitoína é também um fármaco antiepilético, mas não apresenta estes efeitos adversos potencialmente aditivos e nocivos, sendo ainda considerada não inferior em termos de eficácia. É um fármaco cuja administração sistémica pode causar outros efeitos adversos (por exemplo a nível do sistema cardiovascular ou hepático), no entanto a sua administração intranasal poderá aumentar a sua segurança e, potencialmente, também a sua eficácia comparativamente a outras vias não invasivas, no tratamento do *status epilepticus*.

No entanto, a fenitoína é um fármaco de baixa solubilidade aquosa, o que faz com que seja difícil obter uma dosagem elevada sem a utilização de grandes quantidades de solventes orgânicos. Porém, o seu pró-fármaco hidrofílico, a fosfenitoína, tem uma solubilidade aquosa elevada, pelo que poderá ser uma abordagem mais viável para incorporação em formulações líquidas simples, com base aquosa, para administração intranasal. Assim, numa primeira fase do trabalho experimental foram desenvolvidas formulações contendo água, fosfenitoína e um polímero para aumento do tempo de retenção da preparação na cavidade nasal – Pluronic® F-127, um poloxâmero com propriedades termossensíveis, ou hidroxipropilmetilcelulose (HPMC), um derivado da celulose com propriedades viscosificantes e potencialmente mucoadesivas. Foram testadas várias concentrações destes polímeros, sozinhos ou em combinação, e foi selecionada para avançar para estudos farmacocinéticos *in vivo* uma preparação com libertação rápida de fármaco e com potencial mucoadesividade – uma formulação contendo HPMC (dado que, na sua maioria, as formulações contendo Pluronic davam origem a uma libertação prolongada de fármaco). Foi também adicionada albumina à formulação selecionada, por forma a tentar aumentar o direcionamento de fármaco para o cérebro, uma vez que é sabido que a albumina é transportada para o cérebro a partir da cavidade nasal, e que a fenitoína e fosfenitoína têm uma ligação muito elevada a esta proteína. A quantidade de fosfenitoína solubilizada na formulação final foi de 34,8 mg/g equivalentes de fenitoína. Os resultados da administração intranasal em estudos farmacocinéticos em murganhos mostraram que foi quantificável nas matrizes recolhidas (cérebro e sangue) apenas fenitoína, o que indica uma conversão rápida de pró-fármaco em fármaco ativo na cavidade nasal e/ou após absorção. Contrariamente ao esperado, a HPMC não parece ter aumentado a retenção da preparação na cavidade nasal, mas a albumina parece ter prolongado a concentração de fármaco no cérebro, tendo-se obtido uma biodisponibilidade absoluta elevada (comparação com a via intravenosa). Para além disso, os níveis sanguíneos obtidos com as preparações intranasais não foram tão elevados quanto os obtidos com a via intravenosa, o que pode indicar uma maior segurança (menor distribuição sistémica). Neste estudo demonstrámos, então, que os pró-fármacos ésteres de fosfato (como a fosfenitoína) podem ser uma estratégia eficaz no aumento da biodisponibilidade intranasal de fármacos pouco solúveis (como a fenitoína).

Contudo, o facto de existir apenas fosfenitoína na formulação poderia estar a atrasar a chegada de fármaco ao cérebro, já que tinha que existir tempo para ocorrer a conversão a fenitoína (por mais rápida que fosse), e sendo improvável que a fosfenitoína permeasse em grande extensão uma vez que é um composto hidrofílico e duplamente ionizado. Para

além disso, os níveis de fármaco no cérebro aos tempos mais curtos eram ou muito baixos ou nulos (pelo menos com o método analítico aplicado). Pelo que se considerou a hipótese de que a presença de fenitoína na formulação, na forma ativa e pronta a difundir através das barreiras biológicas, pudesse aumentar a rapidez e/ou a extensão com que o fármaco chegava ao cérebro. Posto isto, uma vez que não era possível ter fenitoína em elevada concentração sem recorrer a elevadas quantidades de solventes orgânicos, pensou-se em formular fenitoína em conjunto com fosfenitoína em nano ou microemulsões óleo-em-água, onde a fenitoína estaria internalizada nas gotículas da fase oleosa, e a fosfenitoína solubilizada na fase externa aquosa. Assim, numa segunda fase do trabalho experimental foram desenvolvidas nanoemulsões e nanoemulgéis de formação espontânea, com homogeneização adicional por extrusão por uma membrana de pequeno poro para obtenção de tamanho de gotícula pequeno e homogéneo. A composição do pré-concentrado incluiu o óleo Miglyol 812, o tensioativo hidrofílico Tween 80 e o co-solvente Transcutol (em proporções específicas), mantendo-se uma proporção pré-concentrado/fase aquosa de 40:60 (para maximizar a quantidade de fenitoína) ou 10:90 (para maximizar a dosagem de fármaco total). Daqui obtiveram-se três formulações finais: uma nanoemulsão de viscosidade reduzida e libertação rápida (proporção pré-concentrado/fase aquosa 10:90); e uma nanoemulsão de viscosidade reduzida (proporção pré-concentrado/fase aquosa 40:60) e um nanoemulgel (proporção pré-concentrado/fase aquosa 10:90, com Pluronic na fase externa), ambos com libertação prolongada. No entanto, a dosagem de fármaco atingida não ultrapassou os 22 mg/g ou os 27 mg/g de equivalentes de fenitoína nas nanoemulsões (maior dosagem para menor proporção pré-concentrado/fase aquosa) e 16,7 mg/g de equivalentes de fenitoína no nanoemulgel, o que era mais baixo que a dosagem obtida para as formulações mais simples contendo apenas fosfenitoína. Neste sentido, parecia ainda haver espaço para melhoria e aumentar a concentração de fármaco neste tipo de formulações.

E foi assim que se iniciou a terceira fase do trabalho experimental, na qual se tentou novamente desenvolver nano ou microemulsões contendo fenitoína e fosfenitoína, mas tentando aumentar a dosagem total de fármaco em formulação. Uma vez que tinha dado origem a níveis de fármaco no cérebro prolongados no tempo aquando do estudo farmacocinético com as formulações mais simples de fosfenitoína, a albumina foi assumida como sendo necessária na composição das novas emulsões. Para maximizar a quantidade de fenitoína na forma ativa a quantidade de pré-concentrado foi aumentada, ficando a proporção pré-concentrado/fase aquosa a 50:50. Ainda para aumentar a solubilização de fenitoína na forma ativa (e não como pró-fármaco) foram feitos estudos

preliminares de solubilidade em excipientes individuais. Os excipientes de cada categoria que melhor solubilizavam a fenitoína foram escolhidos e misturados em diversas proporções no pré-concentrado, para que aquando da junção com a fase aquosa dessem origem a nano ou microemulsões com boas características (homogéneas, com tamanho de gotícula pequeno e fisicamente estáveis durante, pelo menos, algumas horas). Foi depois novamente avaliada a capacidade de solubilização de fenitoína, agora nos veículos selecionados, no entanto, apesar do esforço na seleção dos excipientes que melhor solubilizavam o fármaco em questão, a solubilidade da fenitoína foi similar àquela obtida nas nanoemulsões e nanoemulgel previamente desenvolvidos. Assim, por forma a aumentar a dosagem de fármaco (atingindo pelo menos à volta de 34,8 mg/g de equivalentes de fenitoína, o mesmo que nas formulações mais simples de fosfenitoína) aumentou-se a quantidade de fosfenitoína na fase externa. Apenas 2 fórmulas demonstraram compatibilidade com as quantidades requeridas de fenitoína, fosfenitoína e albumina: uma contendo Capryol 90 (óleo), Kolliphor EL (tensioativo hidrofílico) e Transcutol (co-solvente), e outra contendo Capryol 90 (óleo), Kolliphor EL (tensioativo hidrofílico) e Imwitor 988 (tensioativo hidrofóbico), em proporções específicas no pré-concentrado. Estas fórmulas deram origem a microemulsões de tamanho de gotícula pequeno e relativamente homogéneo, com uma viscosidade relativamente baixa. Ambas permitiram uma libertação razoavelmente rápida de fármaco, sendo muito similares entre si. Uma vez que tinham dosagens de fármaco e perfis de libertação semelhantes, selecionámos a microemulsão sem Transcutol para seguir para estudos farmacocinéticos em animais, uma vez que seria potencialmente menos tóxica. Para além da administração intranasal da microemulsão selecionada, contendo fenitoína e fosfenitoína, foi também administrada pela via intranasal uma microemulsão de composição idêntica mas sem fenitoína (só com fosfenitoína), por forma a perceber-se se a existência de uma pequena quantidade de fenitoína na forma ativa e permeável (e não apenas fosfenitoína) é necessária para obter níveis mais elevados ou acelerar a entrega de fármaco ao cérebro. À semelhança do estudo farmacocinético anterior não foi possível quantificar fosfenitoína, apenas fenitoína. Os resultados sugeriram ainda que, apesar da existência de uma pequena quantidade de fenitoína na formulação não ter tornado a entrega de fármaco ao cérebro mais rápida, levou a um prolongamento e aumento dos níveis de fármaco. Adicionalmente, a administração intranasal da microemulsão contendo ambos os fármacos levou a uma concentração máxima no cérebro semelhante àquela obtida aquando da administração intravenosa de fosfenitoína, apesar desses níveis terem demorado mais tempo a serem atingidos, levando também a um prolongamento da retenção de fármaco, o que sugere que a administração intranasal desta microemulsão teve um melhor desempenho que a

solução intravenosa em todos os aspetos exceto na rapidez. A microemulsão contendo ambos os fármacos teve também uma maior eficácia que qualquer outra formulação administrada pela via intranasal contendo apenas fosfenitoína (microemulsão sem fenitoína mas com fosfenitoína, e formulações simples de fosfenitoína), atingindo sempre níveis de fármaco iguais ou (na maior parte dos casos) superiores no cérebro e no sangue. Para além disso, ambas as microemulsões (contendo ambos os fármacos ou apenas fosfenitoína) deram origem a concentrações superiores às obtidas com a solução de fosfenitoína intranasal, o que sugere que os excipientes que faziam parte da composição destas formulações (nomeadamente os agentes tensioativos) tiveram um efeito de aumento da permeação de fármaco.

Assim, globalmente, este trabalho permitiu provar que o uso de pró-fármacos ésteres de fosfato pode ser uma estratégia eficaz no aumento da biodisponibilidade intranasal de fármacos pouco solúveis, que a albumina tem um papel importante no prolongamento dos níveis de fármaco no cérebro (sendo uma boa estratégia de direcionamento cerebral), que a existência de uma pequena quantidade de fármaco ativo (em adição ao pró-fármaco) aumenta os níveis de fármaco a tempos mais longos, e que o uso de agentes tensioativos aumenta a concentração de fármaco a tempos curtos. As estratégias a adotar no futuro, para estes ou outros fármacos, irão depender da intenção: se for apenas a de fazer aumentar os níveis de fármaco pouco tempo após administração, querendo um efeito terapêutico mais rápido mas sem necessidade de o prolongar no tempo, a combinação pró-fármaco + promotor da permeação será a mais favorável; mas se o objetivo for obter níveis elevados mais rapidamente mas também prolongados no tempo, então ter fármaco + pró-fármaco numa emulsão nanométrica + albumina será, das estratégias estudadas, a mais promissora.

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List of abbreviations

ATC	Anatomical therapeutic chemical (classification)
AUC	Area under the “drug concentration vs time” curve
$AUC_{o\text{-}inf}$	Area under the “drug concentration vs time” curve, from time zero to infinity
$AUC_{o\text{-}t}$	Area under the “drug concentration vs time” curve, from time zero to the last quantifiable drug concentration
AUC_{extrap} (%)	Percentage of the area under the “drug concentration vs time” curve that was extrapolated, from the last quantifiable drug concentration to infinity
AUMC	Area under the first moment curve
$B\%_{blood\ IN/IV}$	Absolute blood bioavailability (intranasal formulation vs intravenous solution)
$B\%_{brain\ IN/IV}$	Comparative brain bioavailability (intranasal vs intravenous)
Bias	Deviation from nominal value
C_{last}	Last quantifiable drug concentration
C_{max}	Maximum drug concentration
CV	Coefficient of variation
DTE%	Drug targeting efficiency
DTP%	Direct transport percentage
EDTA	Ethylenediaminetetraacetic acid
FDA	Food and Drug Administration
FOS	Fosphenytoin
FPT	Fosphenytoin, phenytoin and Transcutol aqueous solution
FT	Fosphenytoin and Transcutol aqueous solution
H or HPMC	Hydroxypropyl methylcellulose
HPLC	High-performance liquid chromatography
IN	Intranasal
IV	Intravenous
k_{el}	Elimination rate constant
LLOQ	Lower limit of quantification
MRT	Mean residence time
N	Nanoemulsion
n.d.	Not determined
NS	Not significant (statistical difference)
P	Pluronic F-127
PDI	Polydispersity index
PHT	Phenytoin
QC	Quality control
R^2	Linear regression’s coefficient of determination
$RB\%_{blood}$	Relative blood bioavailability [intranasal formulation (or emulsion) vs intranasal solution]
$RB\%_{blood\ best}$	Relative blood bioavailability (intranasal emulsion vs best intranasal formulation from previous study)
$RB\%_{brain}$	Relative brain bioavailability [intranasal formulation (or emulsion) vs intranasal solution]
$RB\%_{brain\ best}$	Relative brain bioavailability (intranasal emulsion vs best intranasal formulation from previous study)

RDTE%	Relative drug targeting efficiency
RDTP%	Relative direct transport percentage
RMPS	Replicate measurements per sample
r_S	Spearman's correlation coefficient
RT	Room temperature
r_{xy}	Pearson's correlation coefficient
RTmax	Relative Tmax (intranasal nanosystem vs intravenous formulation, or intranasal nanosystem vs intranasal solution)
SD	Standard deviation
SEEDS	Self-emulsifying drug delivery system
SEM	Standard error of the mean
$t_{1/2el}$	Elimination half-life
T_aMax	Maximum acceleration temperature
T_aMin	Minimum acceleration temperature
TG	Thermosensitive gel
T_{gel}50	Half-gelation temperature
Tmax	Time to reach maximum drug concentration
TN	Thermosensitive nanoemulgel
UR	Unfreezing/refreezing
wo	Without

Chapter 1 – Intranasal delivery of antiseizure drugs: an introductory contextualization

1.1. Epileptic seizures

An epileptic seizure is a transient abnormal excessive or synchronous neuronal activity in the brain, resulting in the occurrence of characteristic signs and/or symptoms. Epileptic seizures could be associated with epilepsies, or not. Acute symptomatic seizures (also called situation-related, occasional, reactive, or provoked) can be triggered by high fever (mostly in children), infections of the central nervous system, brain trauma, intoxication, acute hypoglycemia, or many other pathologies [1,2]. Epilepsy is only said to exist if the seizure or seizures are unprovoked and there is a high predisposition to the recurrence of these episodes. For a diagnosis to be established, the patient must have had: at least one unprovoked epileptic seizure, and either a second seizure at least 24 hours apart, or a high risk of recurrence; or diagnosis of an epilepsy syndrome. An epilepsy syndrome is a combination of seizure types, with clinical manifestations and diagnosis results that frequently occur together, as well as characteristic comorbidities [3].

Epilepsy is a high incidence chronic neurological disease (or better said, group of diseases). Active epilepsy was estimated as the third most common neurological disorder in 2017, with a prevalence of about 27 million people, of which 2.5 million were new cases [4]. Even though it predominately affects children and the elderly, it can target all ages. Diagnostic methods include electroencephalography and neuroimaging studies, together with other studies exploring the underlying etiology (medical history of the patient, physical examination, blood and cerebrospinal fluid analysis) [5].

According to the most recent classification guidelines of the International League Against Epilepsy, epileptic seizures can be classified as focal onset, generalized onset, or unknown onset [5]. Generalized seizures represent about 30% of cases and affect both of the brain's hemispheres simultaneously, therefore having more widespread symptoms. Only some seizure types involve involuntary body movements (convulsions), but these are so common that the term "convulsion" is frequently and incorrectly used as a synonym for "seizure", and it is important to distinguish between the two, since "convulsions" are a symptom of (some) "seizures". Focal (also known as partial) seizures, on the other hand, happen in a particular area of the brain, and will consequently have more localized symptoms, representing about 60% of cases. The remaining 10% of cases

are classified as “unknown onset seizures”, which are the ones that the physician cannot classify the nature of the seizure onset [5–8]. Table 1.1 shows the main types of epileptic seizures and their associated symptoms.

Table 1.1. Main types of epileptic seizures and associated symptoms. Adapted from the institutional website for the Epilepsy Foundation [8].

Seizure Type		**Impairment of awareness**	**Symptoms**
Generalized onset	**Tonic**	No, or yes but only partially	Sudden stiffening or tensing of the body, arms or legs.
	Clonic	Yes	Rhythmical jerking of the body or parts of the body.
	Tonic-clonic	Yes	Complete loss of consciousness, muscles stiffening and jerking, person may have trouble breathing and controlling their bladder or bowl.
	Myoclonic	No	Brief shock-like jerks of a muscle or group of muscles.
	Atonic	Yes, at least partially	Sudden loss of muscle tone, head or body may go limp.
	Absence	Yes	Person suddenly stops all activity, starts staring into space, “blanking out”, sometimes mistaken for daydreaming.
Focal onset	**Aware (or simple partial)**	No	Depend on the function of the area of the brain in which they occur; person is fully alert and able to recall events during the seizure, but may or may not be able to respond to others; may experience a sense of *déjà vu*, sensory hallucinations, nausea, or migraine (with aura).
	Impaired awareness (or complex partial)	Yes, at least partially	Depend on the function of the area of the brain in which they occur; person usually becomes unaware of their surroundings and their ability to respond may be impaired; often includes automatic movements; may have an aura; may also include freezing up or wandering, word or phrase repetition, laughing, screaming or crying.
	Focal to bilateral tonic-clonic	Not at first, but yes as the seizure progresses	Person is fully aware in the beginning of the seizure, then loses awareness as it progresses; different phases have different associated symptoms, as described.

Epilepsy can originate from known persisting causes, like traumatic brain injury, stroke, tumor, central nervous system infection, inflammatory or autoimmune disease, and genetic or structural brain abnormalities. Nevertheless, for about half of all epilepsy diagnosis the cause is never found [6,7].

According to the American Epilepsy Society Guideline, epileptic seizure episodes can last less than 5 minutes (brief seizures), between 5 and 30 minutes (prolonged seizures), or more than 30 minutes – *status epilepticus*. *Status epilepticus* is the most severe manifestation of epilepsy. Convulsive *status epilepticus* is its most common type and is also associated with the highest degree of morbidity and, in some cases, even mortality, which can reach up to 30% in adults [9].

1.2. Antiseizure treatment

Treatment depends on the type of seizure, but also on factors that might interfere with the therapeutic outcome and patient compliance, such as the patient's age, other medication, and potential side-effects [7]. In many low and middle-income countries, there is also the issue of drug availability and affordability, thereby limiting the treatment options [10].

To date, there is no cure for epilepsy. Chronic antiseizure treatment is used to control the clinical manifestations of the disease, in order to allow the patients to lead a normal daily life and to prevent the reoccurrence of an epileptic episode. Therapeutic alternatives have become more and more diverse over the years and now include not only pharmacological options, but also implantable medical devices, surgical methods, and dietary modifications. Nevertheless, the first choice of treatment continues to be oral antiseizure (also called antiepileptic or anticonvulsant) drugs [7,11]. The purpose of the antiseizure treatment (rescue or emergency medication) during an acute epileptic episode (such as single seizures lasting more than 5 minutes or seizure clusters) is to end the excessive electrical brain activity as fast as possible, in order to prevent permanent neurological damage [9,12]. There is also evidence that a longer seizure duration is associated with a worst prognosis because the patient becomes less responsive to treatment, mainly due to the internalization of GABA receptors (making GABAergic drugs less effective) or to the up-regulation of drug-efflux transporters [13].

The first-line treatment of convulsive prolonged seizures or *status epilepticus* in hospital setting is usually a parenteral benzodiazepine, namely intravenous lorazepam, intravenous diazepam or intramuscular midazolam. Second-line treatment includes intravenous levetiracetam, lacosamide, phenytoin/fosphenytoin, valproic acid or phenobarbital. Intranasal midazolam or diazepam have also shown to be as effective as the intravenous alternatives. They are also first-line options in pre-hospital setting, along with buccal midazolam or rectal diazepam [9,13].

The non-invasive routes of administration make it possible for epilepsy patients to have these drugs as rescue medication that can be administered by a close relative or other informed person, in case of a breakthrough seizure [12]. Nevertheless, rectal administration has its social barriers, not being well accepted by older children and adults, and buccal administration is associated with the risk of aspiration and a very variable, and many times incomplete, absorption [14,15]. Therefore, in the following sections of this chapter the focus will be on the nasal route of administration.

1.3. Intranasal drug delivery

Nasal anatomy and drug distribution pathways from the nose to the brain have been repeatedly and extensively reviewed over the years [16–18]. Put simply, the central part of the nasal mucosa can be divided into two regions: the respiratory and the olfactory. In the respiratory region, which corresponds to the majority of the surface area, drugs can either enter the systemic circulation, taking advantage of the highly vascularized and permeable epithelium, or diffuse directly to the brain, through the trigeminal nerve pathway. In the olfactory region, located in the upper nasal cavity, drugs can be transported or diffuse directly to the brain through the olfactory nerve pathway (Figure 1.1). Of course, only part of the drug is likely to be absorbed locally, since a fraction might be lost due to enzymatic degradation and mucociliary clearance (being swallowed). Nonetheless, with lipophilic drugs, part of what reaches the systemic circulation will still reach the brain indirectly, by passing through the blood-brain barrier.

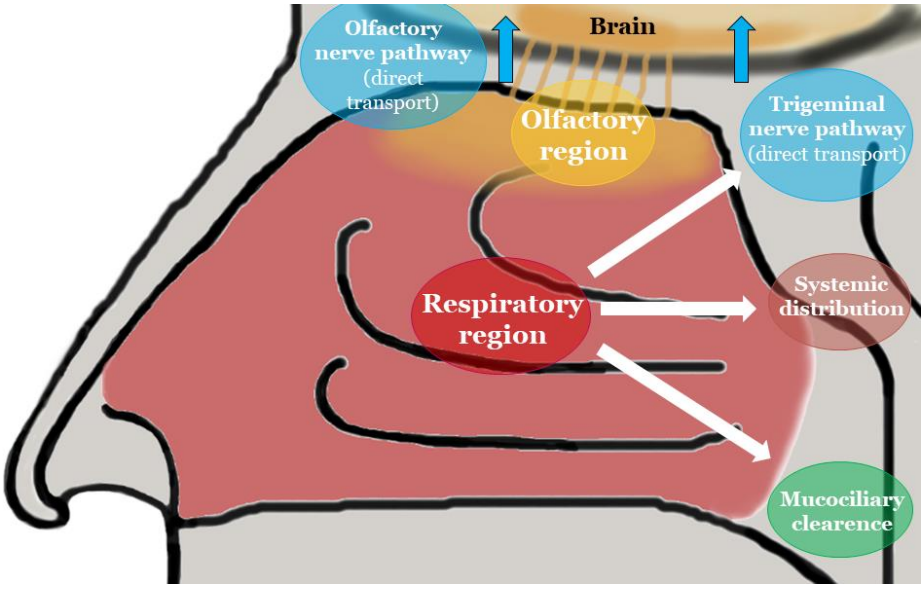

Figure 1.1. Nasal anatomy and drug distribution pathways from the nose to the brain.

Nasal preparations may exist in the form of powders, liquids or semisolids, and should follow some general requirements. For example, none of the components of the formulation should be irritant to the nasal mucosa, and the pH of the liquid preparations (or the pH resulting from powder dissolution) should preferentially be similar to the nasal mucosa's (5.0 to 6.5). Also, isotonic to slightly hypertonic solutions should be used to avoid causing toxicity in the nasal epithelium or enhanced mucociliary clearance. Careful consideration should also be given to the preparation's viscosity and/or particles' bioadhesiveness. In fact, a more viscous/adhesive preparation enhances contact time with the nasal mucosa, which can increase permeation. On the other hand, a highly viscous preparation might also reduce absorption, because of decreased drug diffusion from the formulation itself, or by making it difficult to obtain proper atomization [14,19,20].

1.3.1. Advantages and limitations of intranasal drug delivery

Nasal formulations are naturally advantageous to treat local affections and there are several preparations in the market for that purpose. They are mainly meant for symptoms related to allergies or upper respiratory tract infections, such as nasal congestion, rhinitis and sinusitis [19].

However, there are also several reasons why the development of nasal preparations might be considered for systemic effect drugs, in which many authors agree upon [14,17]. For example, it offers a way to bypass the blood-brain barrier, which is important for drugs that cannot cross it, or that undergo extensive active efflux at this site. Moreover, it is a non-invasive route, not requiring the manufacturing of sterile products. The formulations can be easily administrated by the patients themselves or a caregiver, hence not requiring trained professionals or in-hospital setting. It also offers a short onset of action, being advantageous for the management of emergency situations, which again is good for an outside-of-hospital setting. Furthermore, it does not subject the drugs to the harsh environment of the gastrointestinal tract and first-pass hepatic metabolism, which is critical for peptides, proteins and a great number of other drugs that have a compromised oral bioavailability. It presents itself as well as a suitable alternative for patients in conditions associated with vomiting, increased salivation, or inability to swallow, and it is, as has been previously stated, better accepted by adults and adolescents than the rectal route. The large surface area/volume ratio and high vascularization of the nasal cavity also make it a favorable site for systemic drug absorption. Furthermore, if direct brain transport or diffusion could be granted, nasal

preparations aimed for conditions with a brain etiology could reduce therapeutic drug doses, thereby minimizing systemic adverse effects.

Nevertheless, similarly to all other routes, nasal administration also has its limitations. One of them is that only a low volume can be administered, with the human nasal cavity usually retaining up to 150-200 μL, and therefore requiring a relatively potent drug. The formulation's residence time in the nasal cavity could also be short, due to anterior leakage or posterior drainage, which can limit the time available for drug absorption. Moreover, the presence of degrading enzymes (cytochrome P450 isoenzymes, peptidases, proteases) and efflux transporters (the most common being P-glycoprotein) could also reduce the amount of drug that can be absorbed. Furthermore, it is important that the preparations do not have aggressive odors, and taste sensation might not be avoided. There is the additional risk of pulmonary inhalation (depending on the formula and the administration device), and the administration method and associated device could be critical for drug bioavailability [14,17]. Mucosal inflammation, with nasal obstruction and/or increased mucus discharge, which happens in certain pathological conditions, may likewise pose a problem since it might increase drug absorption variability or reduce the absorption altogether [14].

Initiating a drug and/or formulation development process for intranasal delivery will be worth taking the risk in some cases more than others. The advantages and disadvantages of intranasal drug administration must be weighed together with the challenges presented by each drug and the condition to be treated.

As proof of the potential of intranasal (systemic) delivery, there are also several products on the market already, namely for menopausal symptoms, pain control, endometriosis, migraine headache, and other conditions, as reviewed by Kumar *et al.* [19]. In addition, extensive research exploring the subject of intranasal drug delivery already exists, both with preclinical and clinical studies, as discussed in the following sections.

1.3.2. Strategies to overcome the challenges of intranasal delivery

There are several challenges associated with intranasal drug delivery and over the years scientists have developed several methods to overcome them (Table 1.2). One of the challenges in the delivery of drug molecules through any kind of route is low drug solubility. Several strategies have been used to increase solubility, in either hydrophilic

or hydrophobic formulations. Small lipophilic molecules have the best chance to bypass any biological barrier, but their low aqueous solubility might pose a formulation problem. Moreover, since in intranasal administration the drug has to be administered in a small volume or, if powdered, needs to be dissolved in the aqueous peri-mucosal layer prior to absorption, strategies to increase drug solubility in water have been a major focus [14,17]. This issue can be tackled, for example, by using a water-soluble prodrug that is metabolized in the nasal cavity to produce its parent drug. An example is avizafone, a derivative of diazepam that has a lysine moiety attached to its structure, and can be converted to its parent drug by proteases [14]. Another example is the case of the antiepileptic phenytoin and its hydrophilic prodrug fosphenytoin, in which the prodrug has been shown to convert to the parent drug in the nasal mucosa itself, and both drug forms permeate the mucosa [21].

Table 1.2. Challenges associated with intranasal delivery and strategies to overcome them.

Problem	**Solution**
Low aqueous drug solubility	Water-soluble prodrug
	pH adjustments to increase ionized drug fraction
	Transient supersaturation
	Surfactants or cosolvents
	Nanocarriers
Low drug permeability	Permeation enhancers
	Nanocarriers
Drug degradation	Enzyme inhibitors
	Nanocarriers
Short retention time of the formulation in the nasal cavity	Mucoadhesive excipients
	Viscosity increasing excipients

Increased water solubility can also be obtained by pH adjustments in the formulation, by increasing the ionized drug fraction in solution. If in contact with the neutral pH of the nasal cavity, the drugs will become more lipophilic again, being more easily absorbed across the mucosal membrane. Other strategies to increase drug solubility include the use of transient supersaturation, surfactants, or cosolvents [14]. Another way to formulate drugs and increase their solubility, while also protecting them from enzymatic and chemical degradation, reducing high plasma protein binding, increasing transport through biological membranes, and overall promoting brain bioavailability, is the use of

nanocarriers. This can be particularly beneficial for molecules that, besides having low solubility, also have low permeability [14,22]. Polymeric nanoparticles and lipid-based systems, such as nano and microemulsions or lipid nanoparticles, have been reported to increase drug strength (especially for hydrophobic molecules), while also increasing brain bioavailability when administered through the nasal route. This increased brain bioavailability has been verified, both in comparison with nasal solutions containing the same drugs, and in comparison with the intravenous administration [17].

Drug permeation in the nasal epithelium can also be improved by using permeation enhancers. They can work through several mechanisms: some increase the nasal membrane's fluidity by extracting proteins from it and therefore creating transient hydrophilic pores; others decrease the viscosity of the mucous layer; and others can alter tight junctions. Bile salts are an example of these enhancers, though they have to be used in very small concentrations since they have been reported to be irritant to the nasal mucosa. Some more well-tolerated and safer alternatives are fatty acid salts, borneol, chitosan and cyclodextrins, all having been shown to improve direct nose-to-brain drug transport [6,17].

Enzyme inhibitors could also be used, which is especially useful when the drugs are peptides or proteins, in order to reduce their degradation and hence enhance their potential bioavailability. Some permeation enhancers, such as bile salts and fusidic acid derivatives, are examples of such inhibitors [6].

Another issue with intranasal delivery is the short residence time of the formulation in the nasal cavity. This can be improved by including a mucoadhesive polymer in the formulation, such as pectin, chitosan or sodium alginate, which interacts with the nasal mucosa and helps retain the preparation in the nasal cavity. Another option is to increase the viscosity of the preparation, by using viscosifiers (such as cellulose derivatives) or gelling polymers (such as poloxamers). A solution containing a gelling polymer has the ability to undergo a sol-to-gel phase transition upon contact with the nasal epithelium, triggered by physiological factors such as temperature or the presence of calcium ions. By increasing the preparation's contact time with the nasal mucosa, these excipients potentially lead to an improvement in drug absorption and, consequently, bioavailability [14,17,23,24].

Besides selecting and focusing on the drug intended to be administered and the formulation we want to administer it within, selecting the nasal delivery device and using it properly can also be crucial to obtain the desired outcome. The preparation should be

delivered directly to the surface of the mucosa, and if the amount is too large, or the administration is too fast, there might be a suboptimal absorption and loss of drug into the pharynx, therefore compromising its effectiveness [25]. Nasal solutions dispensed by instillation tend to be cleared more rapidly than those delivered as sprays, since spray tends to deposit on non-ciliated areas of the nasal cavity, while drops are mainly distributed on ciliated surfaces [14,15]. Furthermore, since during a seizure the medication needs to be delivered without active inhalation, sprays or atomized pumps seem to be a good choice, whilst also giving good mucosal distribution [15]. Moreover, innovative devices have been developed and marketed with the aim of achieving direct access to the brain, by channeling the drug to the olfactory region of the nasal cavity (Optinose®, Bi-Directional™ technology) [7]. Additionally, and specifically for the intranasal delivery of antiseizure drugs, a detail that is often overlooked is the nasal mucus production in actively seizing patients, which is usually increased and could, therefore, influence therapeutic efficacy. Suction of mucus from the nasal cavity prior to administration could increase the likelihood of a satisfactory clinical outcome [26].

1.3.3. Preclinical research and brain targeting

Preclinical *in vivo* experiments regarding intranasal drug delivery may be designed with different objectives, and are most frequently performed in rodents, such as mice or rats. They are a way of obtaining proof-of-concept, compare drugs and/or formulation strategies, explore safety, biodistribution and pharmacokinetics, and demonstrate increased brain targeting compared to other administration routes. Brain targeting studies are quite interesting since they usually compare nasal and intravenous administrations, regarding obtained plasma and brain drug concentrations.

However, when interpreting results from animal experiments regarding brain targeting, one should take caution, since these animals possess a proportionally bigger area of olfactory mucosa when compared to humans, being overly optimistic models regarding direct brain delivery pathways.

Preclinical research in intranasal delivery usually comprises four different types of therapies: small drug molecules, peptides and proteins, nucleic acids, or cells. Concerning preclinical studies addressing the intranasal delivery of small drug molecules, cancer, neurological disorders, and psychiatric diseases seem to have been getting the most attention. Chemotherapy has been thoroughly explored, with intranasal delivery being a better way to access brain tumors (methotrexate, raltitrexed, 5-

fluorouracil) [16]. Intranasal delivery of antiseizure drugs in animal models has also been studied to a great extent. For example, several lamotrigine formulations (micelles, micronized powders, thermoreversible gels, nanostructured lipid carriers) have been shown to increase brain drug bioavailability when compared to a nasal solution, or intravenous or oral formulations [6]. Other examples include: carbamazepine, clobazam or levetiracetam microemulsions; clonazepam microemulsions, nanostructured lipid carriers or solid-lipid nanoparticles; diazepam microemulsions or nanoparticles; lorazepam microemulsions or nanoparticles; oxcarbazepine nanoparticles; and valproic acid nanostructured lipid carriers [7,18].

The brain delivery of high molecular weight therapeutics poses an additional challenge, since macromolecular entities are known for their chemical and biological lability [27]. Several animal studies have succeeded in the transport of peptides and proteins to the brain through intranasal administration, such as: peptide-like substances for brain tumors, insulin-like growth factor 1 for the reduction of stroke volume, and nerve growth factor for Alzheimer's disease, depression, and epilepsy [16,17]. Antibodies have also been shown to reach the brain through this route, for the treatment of amyloid angiopathy and plaque pathology in experimental Alzheimer's disease, and for ischemic injury [17]. There have also been a few studies regarding the treatment of epilepsy, namely for the intranasal administration of botulinum neurotoxin A, nerve growth factor, and transforming growth factor beta-1, all of which have shown to have neuroprotective effects [28–30].

Strategies to enhance nucleotides' bioavailability through intranasal administration have been reported as well. Intranasal administration of nanoparticles for gene delivery at remote target cancer cells, or nanoparticles encapsulating pDNA for immunization studies are examples of such [27]. As for the intranasal administration of genetic material as a therapeutic option for epilepsy, there have been a few studies regarding microRNA reducing epileptic seizure frequency, improving seizure onset and reducing hippocampal damage in mice [31,32].

Intranasal vaccination has also been a widely explored area, with antigens being put into a series of different formulations (polymeric or lipidic nanoparticles, liposomes, nanoemulsions, microspheres). Targeted diseases include influenza, tetanus, hepatitis B or meningitis [19,27].

A more recent approach has been the brain delivery of stem cells, immune cells, and genetically-engineered cells through the nasal route, for example for the treatment of

Parkinson's disease, cerebral ischemia, neonatal brain damage, subarachnoid hemorrhage, stroke, intracerebral gliomas and multiple sclerosis [7,16].

Some of the most promising preclinical results have led to clinical trials [17]. As for the administration of antiepileptics, intranasal benzodiazepines have been the focus of many human trials, and three formulations have even reached the market, which will be further discussed in the following section 1.3.4.

Intranasal drug delivery in pre-clinical studies (and the variables that can influence their outcomes) will be further explored in Chapter 2.

1.3.4. Clinical trials

Clinical trials regarding the intranasal administration of antiepileptics for seizure treatment have been entirely focused on the delivery of benzodiazepines, namely clonazepam, diazepam, lorazepam and midazolam [7,14]. In general, clinical trials' results show that intranasal treatments were at least as effective in stopping seizures and preventing their recurrence as the ones that were administered through the intravenous, intramuscular, rectal or buccal routes [25,26,33,34]. Moreover, confirming the general assumption, patients declared being more comfortable with intranasal administration, when confronted with the inconvenience of rectal preparations or the invasiveness of injections, thereby potentially increasing compliance [17]. The preference of intranasal administration as opposed to rectal administration has also been shown by caregivers [15]. In what concerns formulation strategies, drug solubilization was usually achieved by adding high amounts of organic cosolvents to the preparations, such as propylene glycol or polyethylene glycols. These are potentially toxic excipients, and have consequently led to reports of lacrimation, burning and general discomfort in the nose and upper respiratory tract [35–39]. In the case of midazolam a low pH was also necessary for drug solubilization, which also might have caused irritation of the nasal mucosa and upper throat [35–37]. Nevertheless, these effects are overall considered mild, local, reversible and short-lasting, and intranasal administration does not seem to exacerbate active substance-related adverse events.

The first clinical trial regarding intranasal administration of benzodiazepines dates back to 1989 and was a small pharmacokinetic study evaluating innovative formulations of diazepam or lorazepam [40]. As years progressed, the focus moved on to midazolam, which seemed to show more promising clinical results when administered through the

intranasal route than the other benzodiazepines. Nowadays, attention has also turned to diazepam, and what started as an off-label use of intravenous formulations of midazolam or diazepam for intranasal administration, to control epileptic seizures in a non-hospital setting (or in cases of difficult intravenous access in a hospital setting), has led to the development of innovative formulations, with three of them having even already reached the market [26,34].

Nayzilam® is a midazolam nasal spray (5 mg per dose) and was developed by Proximagen, Ltd. It has reached the United States' market, indicated for the acute treatment of seizure clusters. Efficacy in single-dose and long-term uses has been studied and the treatment has been considered to be well-tolerated over an extended period of time, with the maintenance of efficacy suggesting lack of tolerance development [41–47]. Nevertheless, midazolam's solubilization was also achieved by using cosolvents, with the formulation containing, aside from the drug, ethanol, polyethylene glycol-6 methyl ether, polyethylene glycol 400, propylene glycol and water. Hence, as expected, the most commonly reported route specific adverse reactions were nasal discomfort, throat irritation, and rhinorrhea [48,49]. Somnolence was also reported, but as a drug-related issue [47].

Nazolam® is a proprietary patented formulation developed for intranasal administration, also containing midazolam (2.5 or 5 mg per dose). Although it was initially developed for sedation purposes, it is currently used for acute seizure control in the Netherlands, in out-of-hospital settings. Nazolam was concluded to be comparable to intravenous midazolam and better tolerated than Nayzilam, but with similar adverse events (albeit having a lower incidence), since it has ethanol and propylene glycol in its composition [50–52].

Finally, there is Valtoco™, from Neurelis, a diazepam formulation (5, 10, 15 or 20 mg per dose). The vehicle is composed of vitamin E (or similar lipid), ethanol (again, a cosolvent, potentially toxic), benzyl alcohol and an alkyl glucoside [53]. Pharmacokinetic trials showed a very high absolute bioavailability and an improved tolerability profile (compared to previous formulations), with nasal discomfort and alteration in taste sensation being the most common side effects [54–58]. Valtoco's New Drug Application has been accepted in early 2020, with the medication having also already reached the United States' market [59].

Despite favorable results regarding these midazolam and diazepam intranasal formulations, there seems to be scope for improvement regarding formulation

tolerability. Moreover, benzodiazepines are known sedatives, and sedation has been reported as a side effect of these preparations, which also leaves room for wondering about the intranasal administration of other (non-sedative) antiepileptics.

1.4. The case of phenytoin and fosphenytoin

As mentioned above, in a hospital setting, the first-line treatment of convulsive *status epilepticus* is usually an intravenous or intramuscular benzodiazepine, and the second-line treatment includes the intravenous administration of several other antiseizure drugs [9]. Among them is the drug/prodrug pair phenytoin and fosphenytoin, that have had a decrease in use over the years due to systemic side effects (cardiovascular complications, liver toxicity, osteopenia, peripheral neuropathy), but seem to be non-inferior in efficacy when compared to other antiepileptics [9,11,13,60]. In addition, phenytoin is also still widely used in oral form for the chronic treatment of epilepsy, and 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 [61–65]. That being said, by using strategies that could reduce peripheral systemic side effects, such as local or targeted delivery to the intended sites, phenytoin could become a drug of great interest once more [11,60].

The intranasal route could be an alternative to parenteral anticonvulsive drug administration due to several (also already mentioned) associated advantages, and the intranasal administration of phenytoin could increase its efficacy (direct transport to the brain) and safety (reduction of systemic distribution). Also, the intranasal route has already shown its potential in the case of benzodiazepine administration. Furthermore, when compared with benzodiazepines, phenytoin has the advantage of not having their administration route independent side-effects (somnolence, deleterious cognitive effects and dependence/tolerance), which could mean it might be safer in comparison, when administered intranasally.

Nevertheless, phenytoin has a very low aqueous solubility, but its hydrophilic prodrug, fosphenytoin, solves that issue, even though its anionic nature makes it less prone to passive absorption. However, Antunes Viegas *et al.* demonstrated the presence of phosphatase activity within the nasal mucosa, which promotes *in situ* bioconversion of fosphenytoin to phenytoin [21]. Additionally, fosphenytoin could partially permeate porcine nasal mucosa *ex vivo* as prodrug, in addition to the permeation of the neutral parent drug phenytoin, formed by metabolization. Alternatively, one way of formulating

phenytoin would be into a nanosystem with a hydrophobic component, in which the drug could be solubilized. Furthermore, if reaching a high drug strength is not possible, despite efforts to do so, there might be other applications for this drug requiring lower drug strength, such as postsurgical nasal wound healing or trigeminal neuralgia [63,66].

1.5. Thesis' main objectives, tasks and study design

The research work described in the present document had the final goal of serving as proof-of-concept of the hypothesis that phosphate esters can be a useful strategy in nasal formulation development of low aqueous solubility drugs, such as phenytoin, consequently increasing their bioavailability through this route. To achieve that purpose, it included several major tasks, each with their specific objectives:

1. A systematic review of the existing scientific literature, aiming to:
    1.1. Better understand the variables that can influence the outcomes of *in vivo* studies regarding the intranasal administration of small molecular weight drugs within nanosystems;
    1.2. Assess whether there is a specific nanosystem type that is more effective than others in brain drug delivery;
2. Development of liquid and/or semisolid formulations for intranasal administration, containing phenytoin only, fosphenytoin only and/or a drug/prodrug combination, aiming to:
    2.1. Obtain high drug strength, potentially sufficient to be effective in the treatment of *status epilepticus*, or, if not, to serve for other therapeutical applications that may require lower strengths;
    2.2. Achieve formulations with adequate physical/pharmacotechnical attributes, in what concerns viscosity, osmolality, pH, droplet size and surface charge (mean size, polydispersity index and zeta potential, when applicable), and *in vitro* drug release;
3. *In vivo* pharmacokinetic studies aiming to:
    3.1. Compare fosphenytoin solution, mucoadhesive polymer and albumin regarding brain-targeted intranasal drug delivery;
    3.2. Assess whether a combination of drug and prodrug could further improve intranasal drug delivery.

Chapter 2 - Nanosystems in nose-to-brain drug delivery: a review of non-clinical brain targeting studies

2.1. Chapter overview and main objectives

There are many variables that can influence the outcomes of *in vivo* studies comprising intranasal administration. This chapter consists of a systematic review regarding non-clinical studies that evaluated the intranasal delivery of small molecule drugs within nanosystems that were compared to an intravenous formulation (and also in some cases to an intranasal solution), in order to be able to assess possible brain targeting. The present analysis focused on study design, chosen nanosystem class and their characterization, and reported output pharmacokinetic parameters, with emphasis on searching for associations between brain delivery efficacy and nanosystem type. With this analysis we intended to determine which nanoformulation strategy might be better for poorly water-soluble drugs such as phenytoin, with the highest brain targeting and least systemic distribution.

2.2. Methods

2.2.1. Systematic bibliographic selection and data collection

Articles considered for data collection, and consequent statistical analysis, were obtained via search in the "Web of Science™" database, using the following terms: (nanoemulsion$ OR microemulsion$ OR submicron OR emulsion$ OR nanostructured OR miniemulsion$ OR nanoparticle$ OR nanosystem$ OR liposome$ OR cubosome$ OR transfersome$ OR niosome$ OR polymersome$ OR exosome$ OR dendrimer$ OR nanocarrier$ OR micelles) AND brain AND ("in vivo" OR animal OR biodistribution OR pharmacokinetics OR pre-clinical OR non-clinical) AND (nasal OR intranasal). Date of last search was June 23th 2017 and no publication date restriction was applied. Screening for exclusion criteria was done by title, abstract and article content, if needed and in that respective order. Those criteria included: being a review article; not comprising an *in vivo* pharmacokinetic study; absent or insufficient presentation of the necessary pharmacokinetic data, more precisely brain and blood area under the "drug

concentration vs time" curve (AUC) values for (at least) the intranasal nanosystem and the intravenous formulation; inexistence of an intravenous comparison route; drug not having a low molecular weight (namely protein or gene derived entities); intranasal formulation not being a nanosystem; being a vector study only, with no associated drug; being a toxicity study only; lack of information and interpretation quality (repeated errors); and lastly, journal impact factor inferior to 1. Data was extracted to a spreadsheet table format.

2.2.2. Pharmacokinetic ratio's (re)calculation

Given the lack of uniformity or absence of the calculation of drug targeting efficiency (DTE%), direct transport percentage (DTP%), comparative brain bioavailability ($B\%_{brain\ IN/IV}$) and relative brain bioavailability ($RB\%_{brain}$) ratios, whenever possible these were recalculated from reported AUC data.

DTE% is a measure of brain targeting through intranasal administration, with the following mathematical formula:

$$DTE\% = \frac{(AUC_{brain}/AUC_{blood})_{IN}}{(AUC_{brain}/AUC_{blood})_{IV}} \times 100 \quad (2.1)$$

where AUC is the area under the "drug concentration vs time" curve, representing drug concentration variation over time (in brain or blood) for the duration of the study [from time zero to the last quantifiable drug concentration ($AUC_{0\text{-}t}$)], and IN and IV indicate the administration route to which the AUC values correspond to (intranasal and intravenous, respectively) [20,67]. As the given formula implies, the DTE% value can be interpreted as the relative propensity of the drug to accumulate in the brain when administered through the intranasal route, over the intravenous route. Values can range from 0 to +∞. Values above 100% indicate a more efficient brain targeting through intranasal administration when compared to intravenous administration, and values below 100% represent the opposite [67]. The $\log_{10}(DTE\%)$ was also calculated (in attempt to normalize the distributions) and expressed as Log DTE%.

Even if useful, DTE% might not offer an easy interpretation of which drug fraction was transported through the olfactory and trigeminal nerve pathways and which was not [20]. As an alternative, the DTP% can be calculated from equation 2.2:

$$DTP\% = \frac{AUC_{brain_{IN}} - \mathrm{F}}{AUC_{brain_{IN}}} \times 100 \quad (2.2)$$

where AUC values are also $AUC_{0\text{-}t}$, and F is given by:

$$F = \frac{AUC_{brain_{IV}}}{AUC_{blood_{IV}}} \times AUC_{blood_{IN}} \quad (2.3).$$

Since the expected contribution of the indirect pathway to $AUC_{brain\ IN}$ is subtracted from its total value, DTP% is, as the name suggests, the value that better represents the drug fraction undergoing direct transport to the brain [20,67]. Values can theoretically range from -∞ to 100, although the expected value if there is no transport through the direct nose-to-brain pathways is 0. Values higher than 0 indicate the presence of brain targeting through the direct pathways, opposite to values from -∞ to 0, which indicate a more efficient brain targeting through the intravenous route [67]. A value of 100 is only possible if the drug does not cross the blood-brain barrier at all ($AUC_{brain\ IV}$ = 0), or if it is not absorbed to the systemic circulation when administered intranasally ($AUC_{blood\ IN}$ = 0). An approximation to the first case is more likely to occur, and it means that drugs that are poorly permeable in the blood-brain barrier are more likely to have the highest DTP% values. However, DTE% and DTP% can be high despite very low bioavailability in the brain.

$B\%_{brain\ IN/IV}$ is a measure of brain drug accumulation through the intranasal route over the intravenous route, considering brain $AUC_{0\text{-}t}$ values only (and not blood's) [68]. The mathematical formula is:

$$B\%_{brain\ IN/IV} = \frac{AUC_{brain_{IN}}}{AUC_{brain_{IV}}} \times 100 \qquad (2.4)$$

Values above 100 indicate a better brain drug accumulation through intranasal administration when compared to intravenous administration. The $\log_{10}(B\%_{brain\ IN/IV})$ was used and expressed as Log $B\%_{brain\ IN/IV}$.

$RB\%_{brain}$ is similar to $B\%_{brain\ IN/IV}$, only intranasal nanosystem delivery is here compared with an intranasal drug solution [69], the formula being:

$$RB\%_{brain} = \frac{(AUC_{brain_{IN}})_{nanosystem}}{(AUC_{brain_{IN}})_{solution}} \times 100 \qquad (2.5)$$

Values above 100 indicate a better brain drug accumulation with the intranasal administration of the nanosystem when compared to the intranasal solution. The $\log_{10}(RB\%_{brain})$ was used and expressed as Log $RB\%_{brain}$.

The comparison of the efficiency in brain targeting between intranasal nanosystems and intranasal solutions was also verified by calculating the logarithm of relative DTE% (Log RDTE%) and of relative DTP% (Log RDTP%), in analogy with Log RB%. The following formulas where applied:

$$Log\ RDTE\% = \log_{10}\left(\frac{DTE\%_{IN_{nanosystem}}}{DTE\%_{IN_{solution}}} \times 100\right) \qquad (2.6)$$

$$Log\ RDTP\% = \log_{10}\left(\frac{DTP\%_{IN_{nanosystem}}}{DTP\%_{IN_{solution}}} \times 100\right) \qquad (2.7)$$

2.2.3. Statistical analysis

Statistical analysis was performed using Prism software, version 6.0, from GraphPad. Normality of variables distribution was assessed with the D'Agostino-Pearson omnibus normality test. For normal distributions, group mean values were compared with a reference value using the one sample *t*-test (also in the case of insufficient representation, with $n < 5$). In the case of non-normal distributions (and sufficient representation), median values and a Wilcoxon signed-rank test were used. Differences between mean attribute values of nanosystem groups that were at least minimally represented ($n \geq 5$) were evaluated by one-way analysis of variance (ANOVA) with the Tukey multiple comparisons post-test, as were the general mean differences between intranasal solutions, intranasal nanosystems and intravenous formulations (when analyzed as a whole). The overall median differences between intranasal solutions and nanosystems were evaluated by the Mann-Whitney location test. Conservative outlier analysis was performed using the ROUT method (combined Robust regression and Outlier removal) setting Q at 0.1%.

2.3. Results and discussion

2.3.1. Bibliographic search results characterization and quality of reported variables

Of the obtained 243 search results, only 56 met the inclusion/exclusion criteria. Interestingly, the countries of origin were India (66%), Egypt (18%), China (12%), and Saudi Arabia (4%). All articles selected for analysis were published between 2004 and mid-2017, most of them (34%) being published in the year of 2016, and more than half in the last 3 years (2014-2016) (Figure 2.1). Therefore, in recent years there seems to have been a marked increasing interest in the intranasal delivery of nanosystems and

their *in vivo* brain targeting evaluation. Journal impact factors varied from 1.566 to 5.434 (median value of 3.773).

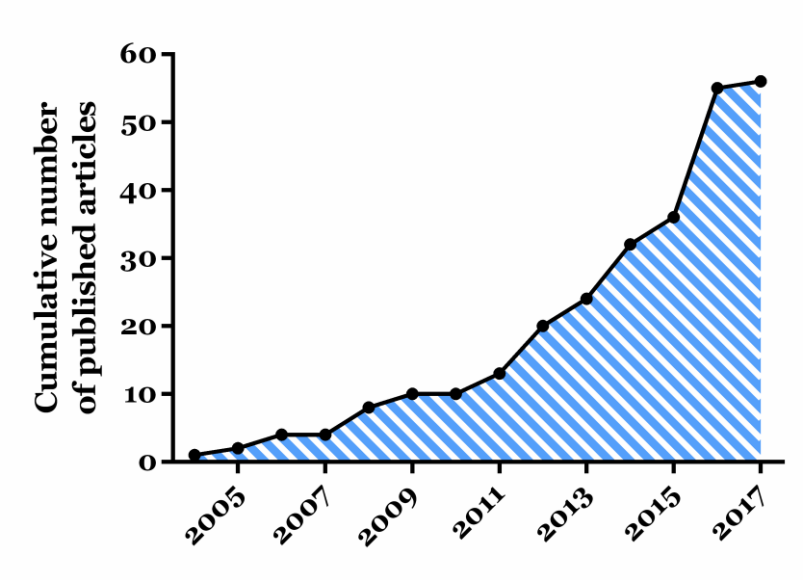

Figure 2.1. Increase in the number of publications on the topic of the present review over time. Data correspond to cumulative publication frequency per year of publication.

Essential parameters such as animal model, study duration, intravenous formulation, intranasal nanosystem type, drug and analytical method were described in every article. On the other hand, analytical method validation was only mentioned in 30% of all publications, while one should expect to find the validation parameters described. Most articles indicated DTE% and most time to reach maximum drug concentration (Tmax) values – $Tmax_{brain\ IN}$, $Tmax_{blood\ IN}$ and $Tmax_{brain\ IV}$. $Tmax_{blood\ IV}$ was only mentioned in 75% of publications because some consider it to be equal to zero, as the drug is readily available in the bloodstream when administered intravenously (a possible reason for other values might be considering $Tmax_{blood\ IV}$ to be the minimum time at which the blood samples were collected). DTP% follows DTE% as the second most reported pharmacokinetic ratio. $B\%_{brain\ IN/IV}$ had a low report rate, and $RB\%_{brain}$ even lower (close to none). Instead, comparative brain AUC fold-changes were sometimes calculated, but they quite similar (ratio instead of a percentage value).

DTE% and DTP%, being ratios that utilize the exact same data, were expected to have a perfect monotonic (non-linear) correlation between them. By examining the scatter-dot plot representations of the correlation between both ratios as mentioned in the articles (Figure 2.2A), it can easily be seen that there was an inconsistency in some of the values

calculated by the authors, with some very strong outliers standing out. Meanwhile, DTE% and DTP% values recalculated by us, using the above-mentioned formulas, had a perfect monotonic correlation (Spearman's correlation coefficient of 1, Figure 2.2B).

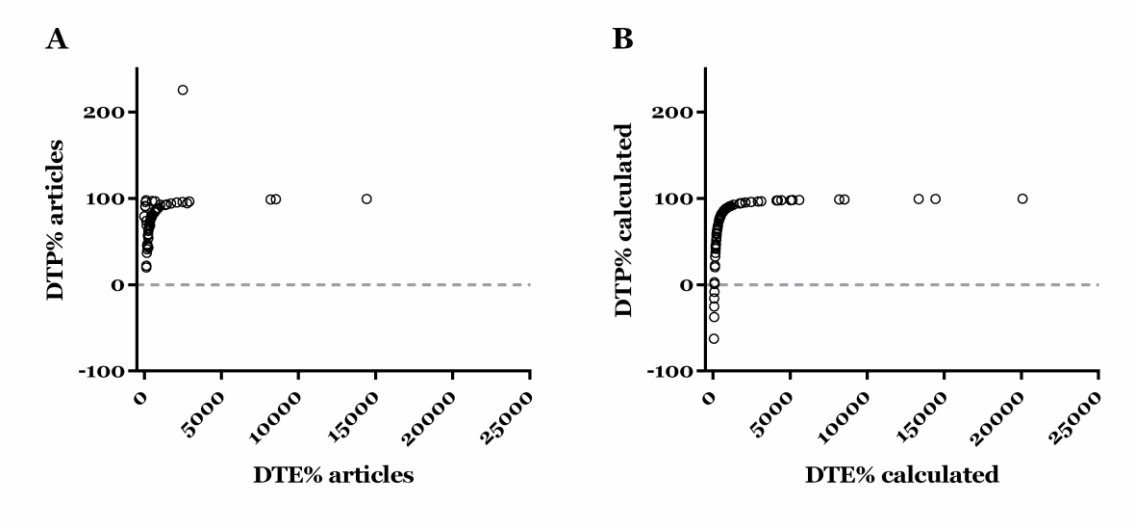

Figure 2.2. Correlation of DTE% and DTP% ratios. Representation of DTE% vs DTP% values as given in the articles (r_s = 0.646) (A) and of DTE% and DTP% when recalculated (r_s = 1.000) (B). DTE% – drug targeting efficiency; DTP% – direct transport percentage; r_s - Spearman's correlation coefficient.

Of all pharmacokinetic ratios, DTE% was the most mentioned, however only 49% of all articles exhibited a value that could be considered equivalent to our recalculated ones. Some of the reasons for the discrepancies in DTE% were: the use of either the area under the "drug concentration vs time" curve from time zero to infinity ($AUC_{0\text{-}inf}$) or a different $AUC_{0\text{-}t}$ value for the calculation, while we used $AUC_{0\text{-}t}$ values corresponding to the duration of the experiment (from time zero to the last measurable concentration); the use of $AUC_{0\text{-}t}$ for calculation, but then only presenting $AUC_{0\text{-}inf}$ values, which we used since we didn't have access to additional data; calculation of DTE% using the $AUC_{0\text{-}t}$ of intravenous nanosystems, while we recalculated them using the $AUC_{0\text{-}t}$ of intravenous solutions (when reported); and the use of a different formula (or non-disclosure of the formula) for the calculation [70–76]. As for (many) other cases, we could find no further clear justification for the revealed discrepancies.

For DTP% and $B\%_{brain\ IN/IV}$, 63% and 35% of the articles reporting these values displayed calculated values similar to ours, respectively. Furthermore, it is relevant to mention that $B\%_{brain\ IN/IV}$ values were either phrased as "absolute brain bioavailabity", "nasal bioavailability" or "comparative bioavailability", and also that it was sometimes hard to determine whether the authors were referring to brain or blood bioavailability, which

made it confusing to interpret. Relative bioavailability values were only provided in 2 articles, both with natural discrepancies in comparison with the ones we calculated, since we used reported AUC_{0-t} and the authors used AUC_{0-inf} [69,77]. All these discrepancies, numeric or not, are evidence of a lack of systematization between studies.

As for formulation attributes, most articles reported nanosystem droplet/particle mean hydrodynamic size (determined by dynamic light scattering) and zeta potential. However, zeta potential is a measure dependent on both particle charge and salt composition of the medium used for particle dilution, and these were not always described. It is often taken as a recommendation that its absolute value should be above 30 mV for maximum stability [78–81]. A direct electrostatic adsorptive interaction with the mucosa is usually expected with high positive zeta potential values, which justifies that chitosan, a natural cationic polymer, is one of the most mentioned mucoadhesive agents [82–84]. The polydispersity index (PDI) was also frequently but not always mentioned, and nanosystems' size is not completely characterized without it [70]. The PDI is also very important in drug pharmacokinetics, since a lower value indicates an enhanced probability of a more uniform absorption through the nasal mucosa, and a higher value may lead to pharmacokinetic irregularity and variability in the therapeutic outcome [85,86]. It is usually recommended that the PDI is below 0.5 [71,78,83]. On the other hand, pH and viscosity were described fewer times. Little more than half of all articles reported formulation pH, and in those that did, it ranged from 4.62 to 7.00, which is fairly within the human nasal mucosa's physiologic range [19]. In addition to having a low reporting rate, viscosity measurements were done at different temperatures (mostly 25 ºC, but also 33 ºC), rotation speeds and spindle types, which makes it hard to interpret and compare between studies. All these formulation attributes can be critical in drug absorption and/or safety, and should be reported in every article.

Other important parameters such as *in vitro* release and *ex vivo* permeation of the drug, although mentioned in some of the articles, will not be considered for this analysis, since only about one half and one third of all publications had, respectively, done these *in vitro* and *ex vivo* studies. Furthermore, reported release and permeation studies' duration, temperature and rotation speed varied substantially from one article to another, or were not mentioned at all, and membrane pore (*in vitro*) and nasal mucosa model (*ex vivo*) also varied greatly.

2.3.2. Delivered drugs

In a total of 56 articles, 39 different drugs were studied, with only one article studying 2 drugs simultaneously (herbal compounds borneol and geniposide, components of the same Chinese traditional medicine) [87]. They belonged most frequently to the antipsychotics, dopaminergic agents, antiepileptics and anxiolytics classes, but many other were represented (Table 2.1). This large heterogeneity, although justifiable by the need to innovate, and therefore to formulate different drugs, makes it difficult to compare, in a direct manner, values of different nanosystems without a great risk of introducing a substantial bias into data interpretation. Even in the case of the most studied drug, olanzapine, an atypical antipsychotic used for the treatment of schizophrenia (which was mentioned in 5 articles) there were 8 different nanosystems: mucoadhesive and non-mucoadhesive nano and microemulsions, polymeric nanoparticles, transfersomes, liposomes and nanocubic vesicular systems. These nanosystems, even if all studied in the same animal model (rat), had different associated analytical methods, intravenous comparison formulations, study durations, doses and excipients [69,81,88–90].

Table 2.1. Anatomical Therapeutic Chemical (ATC) classification of the formulated drugs*, and respective reference.

ATC classification	Chemical entities	Papers (n_i)	Ref.s
A04A Antiemetics and antinauseants	Ondansetron (as hydrochloride)	1	[91]
C04A Peripheral vasodilators	Ergoloid (as mesylate)	1	[92]
C05C Capillary stabilizing agents	Rutin	1	[73]
C08C Selective calcium channel blockers with mainly vascular effects	Nimodipine	2	[93,94]
G03F Progestogens and estrogens in combination	Estradiol	1	[95]
J05A Direct acting antivirals	Saquinavir (as mesylate)	1	[96]
L01A Alkylating agents	Temozolomide	1	[97]
M03B Muscle relaxants	Cyclobenzaprine (as hydrochloride), Tizanidine	2	[82,98]
N02A Opioids	Tramadol	1	[75]
N02C Antimigraine preparations	Sumatriptan, Sumatriptan (as succinate), Zolmitriptan	4	[99–102]
N03A Antiepileptics	Carbamazepine, Clonazepam, Oxcarbazepine	5	[68,77,86,103,104]
N04B Dopaminergic agents	Bromocriptine, Cabergoline, Rasagiline, Ropinirole	6	[85,105–107]
N05A Antipsychotics	Asenapine (as maleate), Haloperidol, Olanzapine, Paliperidone, Risperidone, Quetiapine (as fumarate)	13	[69,72,109–112,78,79,81,84,88–90,108]
N05B Anxiolytics	Alprazolam, Buspirone (as hydrochloride), Clobazam, Diazepam	5	[83,113–115]
N06A Antidepressants	Duloxetine, Venlafaxine	3	[80,116,117]
N06D Anti-dementia drugs	Rivastigmine, Tacrine (as hydrochloride)	2	[71,118]
P01B Antimalarials	Artemether (as artemisinin methyl ether)	1	[119]
Experimental	Resveratrol, Tarenflurbil	2	[76,120]
Plant derivatives	Borneol, Curcumin, Demethoxycurcumin, Bisdemethoxycurcumin, Geniposide, Thymoquinone	5	[74,87,121–123]

n_i, - absolute frequency; Ref.s – bibliographic references; * all ATC classification obtained from the DrugBank database, Version 5.0.7 [124]

2.3.3. Delivery nanosystems

From the 56 articles that were analyzed, 31 focused on the study of one formulation of one nanosystem type only, while 25 studied two or more. Reasons for studying more than one formulation included, most frequently, the addition of a mucoadhesive agent, but also other variations in constituents (different surfactants, PEGylation, variation of polymers' molecular weight, addition of cyclodextrins). Fewer times, there was also the comparison of two nanosystem types, or drugs (two different drugs, or chemical derivatives of the same drug).

We classified the formulations using a two-level classification system (Table 2.2). On level I we grouped them in a minimum number of nanosystem types: emulsions, polymeric nanosystems, lipid nanoparticles, and liposome related nanosystems. On level II, the detail in nanosystem distinction was increased, using a classical class definition. Rare nanosystems, containing phospholipids in their composition, occurring one time only, were grouped in the same class and named "other" (niosomes, nanocubic vesicular systems, emulsomes and lipidic micelles).

Table 2.2. Frequency distribution of formulations by nanosystem class using a two-level classification system.

Level I - groups	**n_i**	**Level II - classes**	**n_i**	**Formulations compared to intranasal solutions* (n_i)**
Emulsions	50	Microemulsions	36	26
		Nanoemulsions	14	12
Polymeric nanosystems	26	Polymeric nanoparticles	21	19
		Polymeric micelles	5	5
Lipid nanoparticles	8	Solid lipid nanoparticles	3	2
		Nanostructured lipid carriers	5	4
Liposome related nanosystems	10	Liposomes	4	0
		Transfersomes	2	0
		Other	4	0
n	94		94	68

n - total number of events; n_i - absolute frequency; * or drug dispersions (one case).

Regarding the frequency of formulation types in the analyzed data, emulsions were the most studied delivery nanosystem group, comprising more than half of all formulations (Table 2.2). Within that group, microemulsions were the most studied class.

Nanometric emulsions (colloidal liquid in liquid dispersions) include nanoemulsions and microemulsions, not always clearly distinguished in the scientific literature. Theoretically, while the first have thermodynamic stability, the later do not. Nanoemulsions have, nonetheless, a relatively high kinetic stability [75,103,111], a higher surface area and higher free energy than macroemulsions, being more stable against sedimentation, flocculation, coalescence and creaming [76]. Given that water is added to a mixture of oil, surfactant and cosurfactant, both types of emulsions are said to form spontaneously [19,87]. Droplet size range is generally considered to be lower in microemulsions (10-100 nm) than in nanoemulsions (20-200 nm). Another definition is simply classifying a system as a microemulsion when it is translucent, which is questionable since it only means that most droplets are very small in diameter, and partially translucent systems could either be macroemulsions or nanoemulsions. Their lipophilic nature, good permeability and solubilizing effect also make them promising systems for intranasal delivery, in particular for liposoluble drugs [75,78,81,85,96]. However, a few reports have stated irritation of gastrointestinal or nasal mucosa with the use of these preparations, justified by the existence of surfactants in large amounts [92,101]. They could also undergo rapid nasal clearance, but a mucoadhesive agent can be added to the formulations to overcome mucociliary clearance, leading to a higher residence time at the site of absorption, and therefore improving bioavailability [82,83].

Polymeric nanosystems followed emulsions in frequency, polymeric nanoparticles being the most frequent class in that group (Table 2.2). Polymeric nanoparticles are compact colloidal systems with a highly variable size range within the nanometric scale, composed of natural or artificial polymers [19,83]. Drugs will be dissolved, entrapped, encapsulated or attached to the polymeric matrix [83,113]. Surface hydrophobicity, high drug loading and controlled release capacity, and the ability to prolong the duration of therapeutic effect, are a few of their advantages. As in the case of mucoadhesive nano and microemulsions, the integration of mucoadhesive polymers into the polymeric nanoparticles formulation is expected to lead to a higher residence time on the nasal mucosa [82,83]. Although there is scientific evidence suggesting the biodegradability and biocompatibility of the polymers that are generally used, some reports mention toxicity, and also formation of aggregates with a large size and lack of stability in aqueous dispersion, leading to phase separation [100,114].

Even if made of polymers as well, polymeric micelles differ in composition from polymeric nanoparticles: they are comprised of amphiphilic block copolymers that will self-assembly, forming a hydrophobic core, and a hydrophilic corona. The core can solubilize and incorporate a lipophilic drug, while the hydrophilic corona will serve as a

stabilizing interface between the hydrophobic core and the external aqueous environment. This kinetic stability and self-assembly in water will only occur above the critical micelle concentration, which is, however, usually lower than that of small molecule surfactants. Described drawbacks include the formation of aggregates with a large particle size and lack of stability in aqueous dispersion, resulting in phase separation [69,77,94].

Within the lipid nanoparticles group, the best known are perhaps the solid lipid nanoparticles, which are solid matricial nanoparticles made of solid lipids dispersed in water or an aqueous surfactant solution [114]. They are said to have increased drug stability, good biocompatibility and tolerability, and high drug loading, also increasing nasal retention time due to an occlusive effect and mucous membrane adhesion [109,114]. Despite many claims of controlled delivery of hydrophobic drugs, some reports have reached opposite results, which authors explain happens because drug and lipid solidification in phase-separated crystals precipitate, either in the core or on the surface of the nanoparticles, with a consequent slow or pronounced burst release [68]. Leakage during storage by lipid polymorphism has also been mentioned [79,91]. In their turn, instead of only having a solid lipid in their composition, nanostructured lipid carriers have a blend of solid and liquid lipids that form an imperfect crystal matrix in which drugs can be accommodated in [19,119]. General advantages include rapid uptake, absence of burst effect and good tolerability. Stated advantages over polymeric nanoparticles comprise avoidance of the use of organic solvents in production, and over solid lipid nanoparticles higher drug loading, smaller particle size and no drug leakage or expulsion during storage, with improved long-term stability [79,91,97,117,119,125].

Within the liposome related nanosystems group, classic liposomes were the most represented class. These are biocompatible and biodegradable vesicles composed of phospholipid (and cholesterol) bilayers enclosing one or more aqueous compartments [19,22]. Several variations of these particles have been developed. In transfersomes the lipidic bilayers' specific composition and membrane incorporated edge activators give the vesicles high flexibility, making it easier for them to interact with the membranes and pass through small fenestrations [90]. Niosomes are prepared with non-ionic surfactants such as monoester of polyoxyethylene fatty acids, free fatty acids and cholesterol, and have a high capacity central core that can deliver a large volume of active ingredient [100]. Nanocubic vesicular systems have polymeric non-ionic surfactants integrated in their phospholipidic bilayer, whose very specific ratio in relation to the phospholipid gives the vesicles cubic shape [89]. Emulsomes have the combined characteristics of emulsions, solid lipid nanoparticles or nanostructured lipid carriers, and liposomes: a lipidic core in a

solid or liquid crystalline state (instead of an oil fluid phase), surrounded by at least one phospholipid bilayer envelope, with an aqueous interface in between (hydrophilic heads facing outwards and hydrophobic tails facing inwards). Emulsomes allow high loads of lipophilic drugs and a prolonged release time. When compared to other lipid carriers, such as solid lipid nanoparticles and liposomes, these particles are described as not showing the common preparation methods' shortcomings, such as high pressure induced drug degradation, lipid crystallization, gelation and coexistence of several colloidal species [68]. Lastly, lipidic polymeric micelles are, just like regular micelles, made of amphiphilic block copolymers, but those copolymers are now also attached to a phospholipid, which generates a more lipophilic, although larger, nanosystem [94].

Additionally, it is relevant to mention that in the majority of studies (about 72% of all articles) the nanosystems were compared to the respective drug solutions or, in one case, drug dispersion (Table 2.2).

2.3.4. Pharmacokinetic study designs and drug assays

Only two animal models were used: rat (75%) and mice (25%). Study duration varied substantially, ranging between 2 and 72 hours, with a median value of 8 hours, which was also the duration of almost half of all studies. Every study used the intravenous route as a parenteral comparison route, although not all animal subjects were given an intravenous drug dispersion, with 39% of all articles administering the nanosystem itself. This is a major inconsistency, since the pharmacokinetic profile of the intravenously administered drug, in solution or associated to a nanosystem, can be quite different, and the calculation of DTE%, DTP% and $B\%_{brain\ IN/IV}$ of the intranasal formulation should be made using intravenous drug solution data. For drug assay, the most utilized analytical method was liquid chromatography (57%), which included high and ultra-performance systems, and was often coupled with a tandem mass spectrometry detector, followed by scintigraphy (43%).

2.3.5. Nanosystem attributes

In what concerns mean particle size (Figure 2.3A), the most homogeneous formulations, with the smallest value range, were the microemulsions. They also had the lowest mean value, which was significantly lower than that of the polymeric nanoparticles. Polymeric nanoparticles had the highest range, having the most heterogeneous particle size

distribution out of the 4 groups, which might be explained by the different subtypes of nanoparticles and their respective composition and preparation methods. Polymeric nanoparticles also had a significantly higher mean than the polymeric micelles and the nanoemulsions classes (aside from the microemulsions). All these results are in agreement with these nanosystems' theoretical size definition.

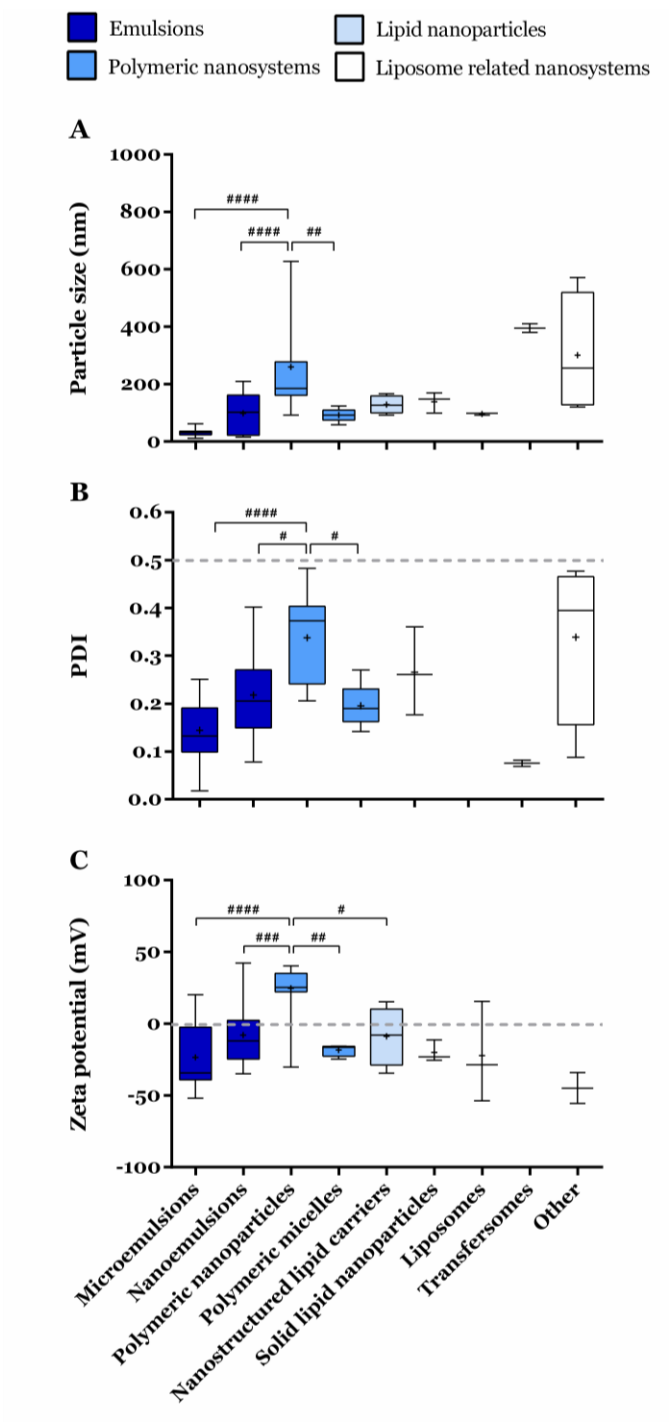

Figure 2.3. Summary of the attributes of the drug delivery nanosystems per class. Particle size (A), PDI (B) and zeta potential (C) are shown. Data correspond to median ± inter-quartile interval and range (box-plot) plus mean indicated by a small "plus" sign (+). Statistical analysis was done by applying a one-way ANOVA with the Tukey multiple comparisons post-test; # $p < 0.05$; # # $p < 0.01$; # # # $p < 0.001$; # # # # $p < 0.0001$. PDI – polydispersity index.

Homogeneity of particle size distribution is characterized by PDI values (Figure 2.3B), of which microemulsions had the lowest mean and polymeric nanoparticles the highest. The difference between means of these two classes was significant, and the mean of the polymeric nanoparticles class was also significantly higher than that of the polymeric micelles and nanoemulsions classes, which is in accordance with what has been mentioned above. All PDI values were below the recommended 0.5 threshold.

As previously mentioned, zeta potential values can either be positive or negative, depending on the combined charges and quantities of the drugs and excipients that compose the formulation. Positively charged formulations have, in theory, the advantage of interacting with the negatively charged nasal mucosal membrane due to the formation of electrostatic bonds with the sialic acid residues that are part of its composition. This facilitates both adhesion and transport by increasing contact time with said region and amplifying the opening of the tight junctions that exist there [70,73,125]. About half of all nanosystem groups included negatively charged nanosystems only, and for those that comprised both positively and negatively charged formulations, most values were also negative (Figure 2.3C). Out of the 5 considered classes, microemulsions had the most negative mean value, and polymeric nanoparticles the most positive, with the differences between the two classes being significant. Polymeric nanoparticles also had significant differences when compared to the polymeric micelles, nanoemulsions and nanostructured lipid carriers' classes. The more positive values attributed to the polymeric nanoparticles class might be justified by the frequent presence of the positively charged polymer chitosan in their composition.

2.3.6. Drug pharmacokinetics

The median values of Log DTE%, DTP% and Log $B\%_{brain\ IN/IV}$ of all nanosystem formulations grouped together was superior and significantly different from the median values of the 42 solutions that were also evaluated (Figures 2.4A, B and C). Furthermore, their value range was extremely high, not only in the nanosystems group, where one could already expect it, but also in the solution group. This big variability in drug delivery performance of intranasal solutions can only be due to either the drug itself or confounding variables related with study design, such as differences in study duration and administered dose, both of which can greatly influence the AUC values and, consequently, their ratios. Even a conservative outlier analysis (ROUT, Q = 0.1%) identified several possible outliers. The very high Log DTE% values in the solution group (Figure 2.4A) corresponded to plant derived drugs [73,74], and are probably due to their

high hydrophilicity and consequent difficulty in permeating the blood-brain barrier (low brain bioavailability through the intravenous route). Some very low DTP% values (negative or close to 0, Figure 2.4B) correspond to the nanosystems group, more specifically to risperidone liposomes, olanzapine liposomes, olanzapine transfersomes and olanzapine nanocubic vesicular systems (3 independent studies [89,90,110]). Curiously, all these formulations belong to the liposome related nanosystems group.

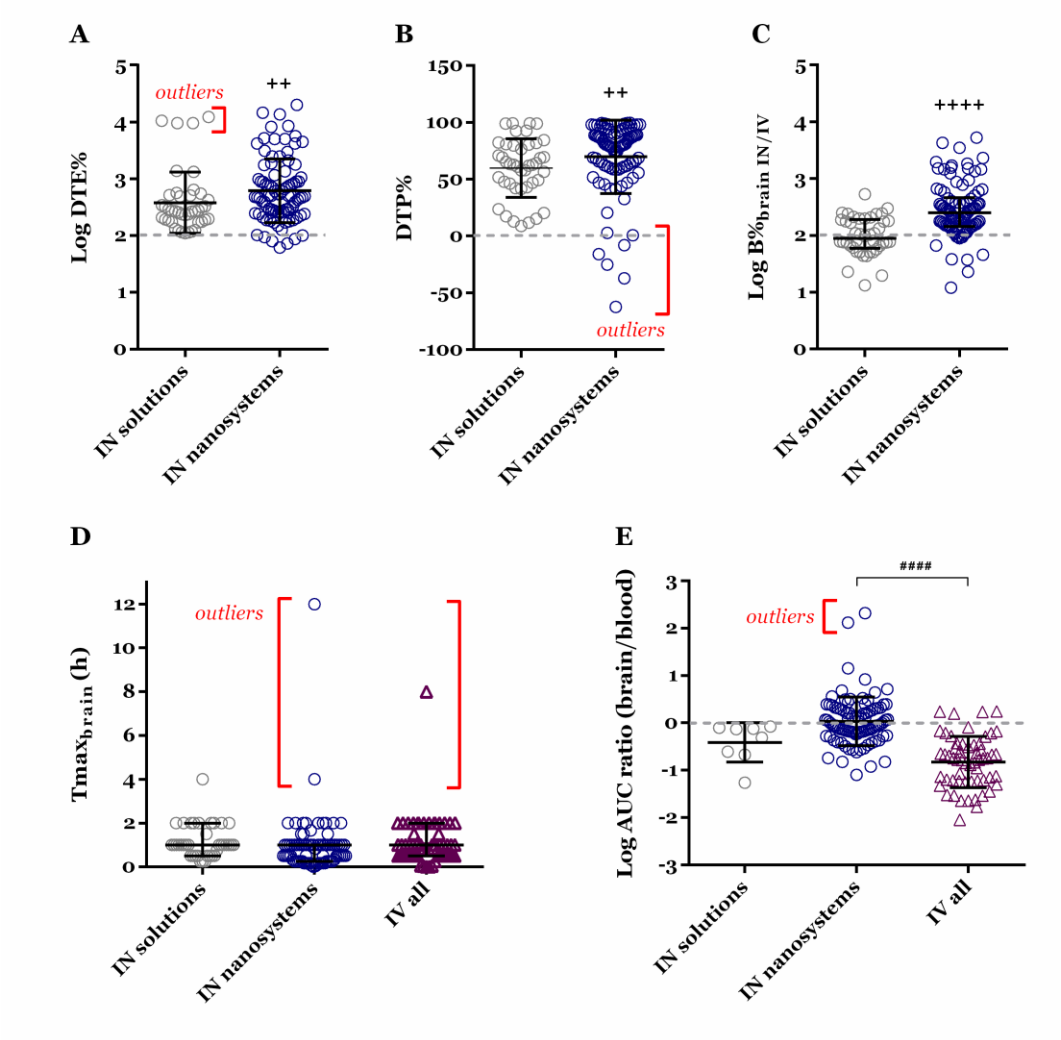

Figure 2.4. Comparison of overall drug delivery by nanosystems and solutions. Log DTE% (A), DTP% (B), Log $B\%_{brain\ IN/IV}$ (C) of intranasal solutions and intranasal nanosystems, $Tmax_{brain\ IN}$ (D) and Log AUC ratio (brain/blood) IN of intranasal solutions, intranasal nanosystems and all intravenous formulations are shown. Potential outliers are signaled by brackets. Data correspond to individual values plus median ± quartiles. Statistical analysis was done by applying Mann-Whitney U test when comparing intranasal nanosystems to intranasal solutions (control), + + $p < 0.01$, + + + + $p < 0.0001$; and by applying one-way ANOVA with the Tukey multiple comparisons post-test when comparing intranasal nanosystems, intranasal solutions and intravenous formulations, # $p < 0.05$, # # $p < 0.01$, # # # $p < 0.001$, # # # # $p < 0.0001$. $B\%_{brain\ IN/IV}$ – comparative brain bioavailability (intranasal vs intravenous); AUC – area under the "drug concentration vs time" curve; DTE% – drug targeting efficiency; DTP% – direct transport percentage; IN – intranasal; IV – intravenous; Tmax – time to reach maximum drug concentration.

In the matter of the amount of time it takes the drug to reach maximum concentration in the brain ($Tmax_{brain\ IN}$), three possible outliers were identified (Figure 2.4D): one intranasal zolmitriptan nanoemulsion, one intranasal thymoquinone polymeric nanoparticles formulation, and one intravenous thymoquinone polymeric nanoparticles formulation, all having Tmax values of 4 hours or greater [101,121]. In the case of the intranasal zolmitriptan nanoemulsion, the obtained Tmax might be justified by the fact that the authors quantified the drug in the cerebrospinal fluid, instead of the brain (which is what was done in most articles), a compartment that the drug may take more time to reach. In the case of the thymoquinone preparations, the high Tmax value could be due to a slow drug release from the nanosystem, as suggested by the release studies performed for those same formulations in the considered article.

Log AUC ratio (brain/blood) also had a couple of extremely high values identified as possible outliers (Figure 2.4E), both corresponding to the intranasal geniposide microemulsions [87]. These high values might be justified by the drugs' rapid elimination from the blood, leading to low blood AUC [126,127].

The parameters DTE%, DTP% and $B\%_{brain\ IN/IV}$ showed an ample value range within each nanosystem class, with DTE% reaching 4 digits (Figures 2.5A, 5C and 5E). This high variability could be due to several factors, such as nanosystems' composition (both drug and excipients used) and their properties, in addition to confounding variables (study design), as discussed. Considering only the most represented nanosystem classes, all mean/median values of Log DTE%, DTP% and Log $B\%_{brain\ IN/IV}$ were significantly higher than the respective reference values (0 for DTP%, 2 for Log-transformed ratios). The highest Log DTE% mean/median value belonged to the polymeric micelles class, being significantly different from the Log DTE% of microemulsions (one-way ANOVA). This class also had the second highest Log $B\%_{brain\ IN/IV}$ mean value, which was significantly better than the microemulsions class, and, even though no statistical significance was found, the highest DTP%. Microemulsions had some of the lowest mean/median values, having a significantly lower $B\%_{brain\ IN/IV}$ than polymeric nanoparticles and nanostructured lipid carriers, besides polymeric micelles (one-way ANOVA).

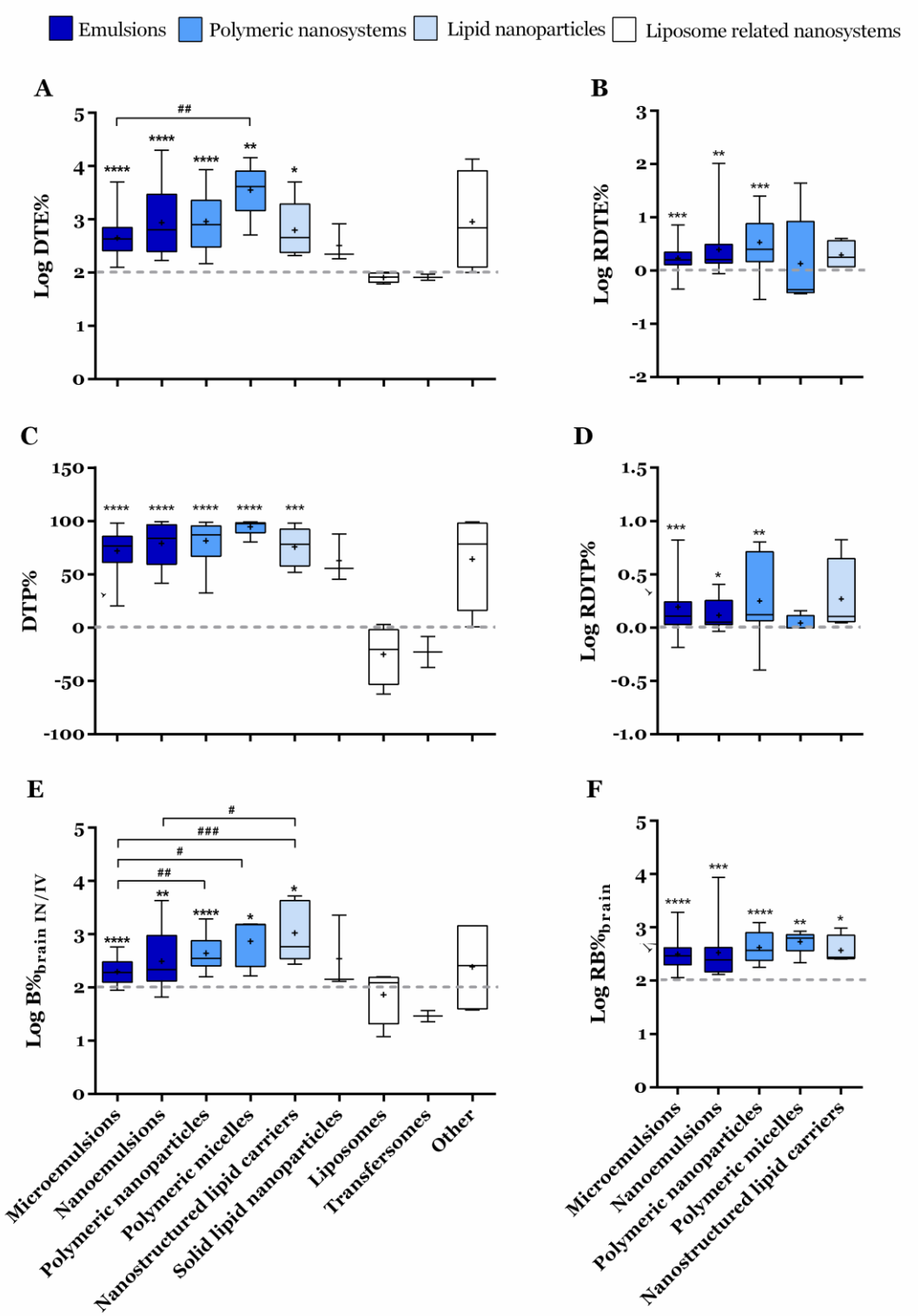

Figure 2.5. Formulations' brain targeting and bioavailability summary. Log DTE% (A), Log RDTE% (B), DTP% (C), Log RDTP% (D), Log B%$_{\text{brain IN/IV}}$ (E) and Log RB%$_{\text{brain}}$ (F) are represented for the different formulation classes. Data correspond to median ± inter-quartile interval and range (box-plot) plus mean indicated by a small "plus" sign (+). Statistical significance of differences between group means evaluated by one-way ANOVA with the Tukey multiple comparisons post-test, # $p < 0.05$, # # $p < 0.01$; # # # $p < 0.001$; differences between means and reference "no-change" values by one-sample *t*-test when normal distribution, Wilcoxon signed-rank test when not (medians), * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. B%$_{\text{brain IN/IV}}$ – comparative brain bioavailability (intranasal vs intravenous); DTE% – drug targeting efficiency; DTP% – direct transport percentage; RB%$_{\text{brain}}$ – relative brain bioavailability (intranasal nanosystem vs intranasal solution); RDTE% – relative drug targeting efficiency; RDTP% – relative direct transport percentage.

It may strike the eye that liposomes and transfersomes perform poorly, and they were already discussed as possible outliers. However, all liposomes and transfersomes data came from only two articles, therefore possibly being isolated cases of poor performance.

Furthermore, the direct comparison of nanosystem behavior to the respective drug solution could highlight the advantage of using nanosystems, eliminating the effect of drugs and confounding variables. To do so, we compared DTE% and DTP% values of the intranasal nanosystems and the respective intranasal solutions (only studies comparing both were included) by calculating their relative values (RDTE% and RDTP%) plus $RB\%_{brain}$, all Log-transformed (Figures 2.5B, 5D and 5F). In the great majority of cases, the intranasal nanosystem had better brain drug targeting and direct transport than the respective solution. Furthermore, microemulsions, nanoemulsions and polymeric nanoparticles classes mean/median values were significantly different from zero (Figures 2.5B and 5D, one sample *t*-test when normal distribution, Wilcoxon signed-rank test when not). Relative bioavailability was also improved in all groups (Figure 2.5F, same statistical analysis). Nevertheless, we should not consider brain values only, since high blood values can lead to undesirable systemic side effects. Differences between groups were not statistically significant (one-way ANOVA).

Taking this analysis into account, among the groups where the analysis had statistical power, microemulsions were the ones that performed less well (even if still having an improved brain targeting in comparison with the intravenous formulation). Polymeric micelles seemed to be the most successful when considering Log DTE% values. However, in this last class the evaluation of the relative brain targeting (both Log RDTE% and Log RDTP%, Figures 2.5B and 5D) failed to show an evident improvement over the respective intranasal drug solutions. This was due to the fact that some of the solutions used in these studies performed surprisingly well, and some constituted possible outliers in the solutions data set, as previously mentioned (Figure 2.4A). Therefore, the drug itself seems to noticeably influence the brain targeting efficiency. In contrast, the increase of relative brain/blood bioavailability seems less dependent on the drug, and more on the achievement of the nanosystems themselves. In fact, Log DTE% correlated better (and negatively) with $Log(AUC_{brain}/AUC_{blood})_{IV}$ than it did (positively) with $Log(AUC_{brain}/AUC_{blood})_{IN}$, which indicates that good DTE% (and, consequently, DTP%) values were mostly due to a low intravenous brain/blood AUC ratio than due to a high intranasal brain/blood AUC ratio (Table 2.3 and Figure 2.6). That could be attributed to the studied drug's properties, and its poor blood-brain barrier permeability. Moreover, the same correlations happen with $B\%_{brain\ IN/IV}$, but in a lesser extent, further confirming

the influence of intravenous brain AUC values (that are not included in this ratio's calculation).

Table 2.3. Spearman and Pearson correlations of Log DTE%, Log $B\%_{brain\ IN/IV}$ and logarithm values of relative bioavailability through the intranasal and intravenous routes.

		Log DTE%	Log $B\%_{brain\ IN/IV}$
Log (AUCbrain/AUCblood)$_{IN}$	**r_s**	0.24	0.23
	Spearman *p* value	0.023	0.022
	r_{xy}	0.29	0.13
	Pearson *p* value	0.023	0.022
Log (AUCbrain/AUCblood)$_{IV}$	**r_s**	-0.76	-0.44
	Spearman *p* value	< 0.0001	< 0.0001
	r_{xy}	-0.73	-0.47
	Pearson *p* value	< 0.0001	< 0.0001
Log $B\%_{brain\ IN/IV}$	**r_s**	0.68	-
	Spearman *p* value	< 0.0001	-
	r_{xy}	0.68	-
	Pearson *p* value	< 0.0001	-

AUC – area under the "drug concentration vs time" curve; $B\%_{brain\ IN/IV}$ – comparative brain bioavailability (intranasal vs intravenous); DTE% – drug targeting efficiency; r_s – Spearman's correlation coefficient; r_{xy} – Pearson's correlation coefficient.

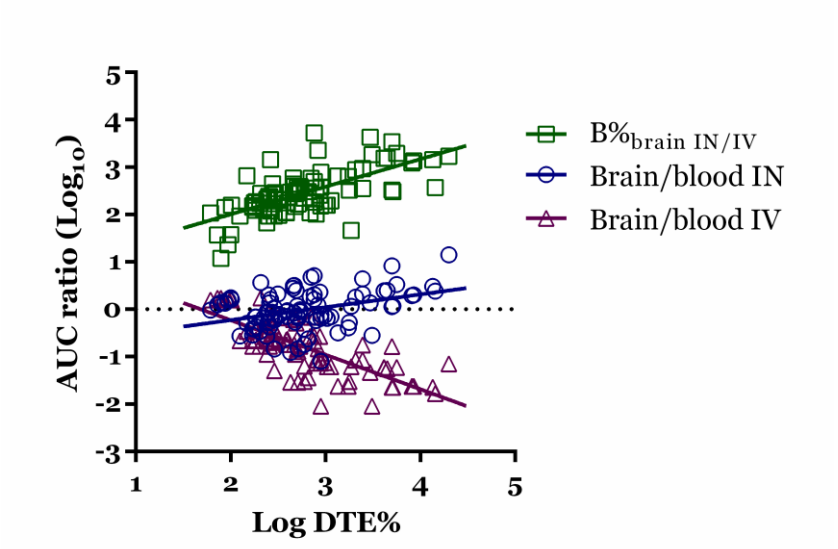

Figure 2.6. Graphical representation of the correlation of Log DTE% with Log $B\%_{brain\ IN/IV}$ and Log AUC ratios; AUC – area under the "drug concentration vs time" curve; $B\%_{brain\ IN/IV}$ – comparative brain bioavailability (intranasal vs intravenous); DTE% – drug targeting efficiency; IN – intranasal; IV – intravenous.

Tmax values' distribution of each nanosystem class was not able to discriminate significant differences among nanosystems (ANOVA, Figure 2.7).

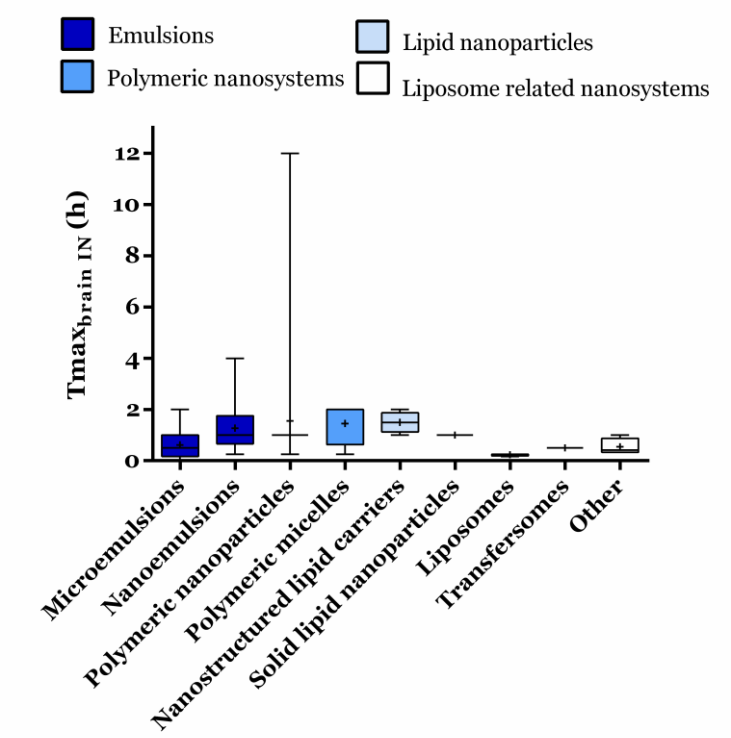

Figure 2.7. Representation of $Tmax_{brain\ IN}$ values for all nanosystem groups. Data correspond to the median ± inter-quartile interval and range (box-plot), and the mean, indicated by a small "plus" sign (+). IN – intranasal; Tmax – time to reach maximum drug concentration.

In what concerns relative values, most mean/median values of log transformed relative Tmax in the brain (Log $RTmax_{brain}$) of the intranasal nanosystems compared to the intravenous route (Figure 2.8A), and of the intranasal nanosystems compared to the respective intranasal solutions (Figure 2.8B), are equal to or below zero, with the small gains only reaching statistical significance in the most represented class, microemulsions. This indicates that brain delivery through the intranasal route can be at least as fast as the intravenous route, making it a good option when there is a need for the time to achieve therapeutical effect to be short.

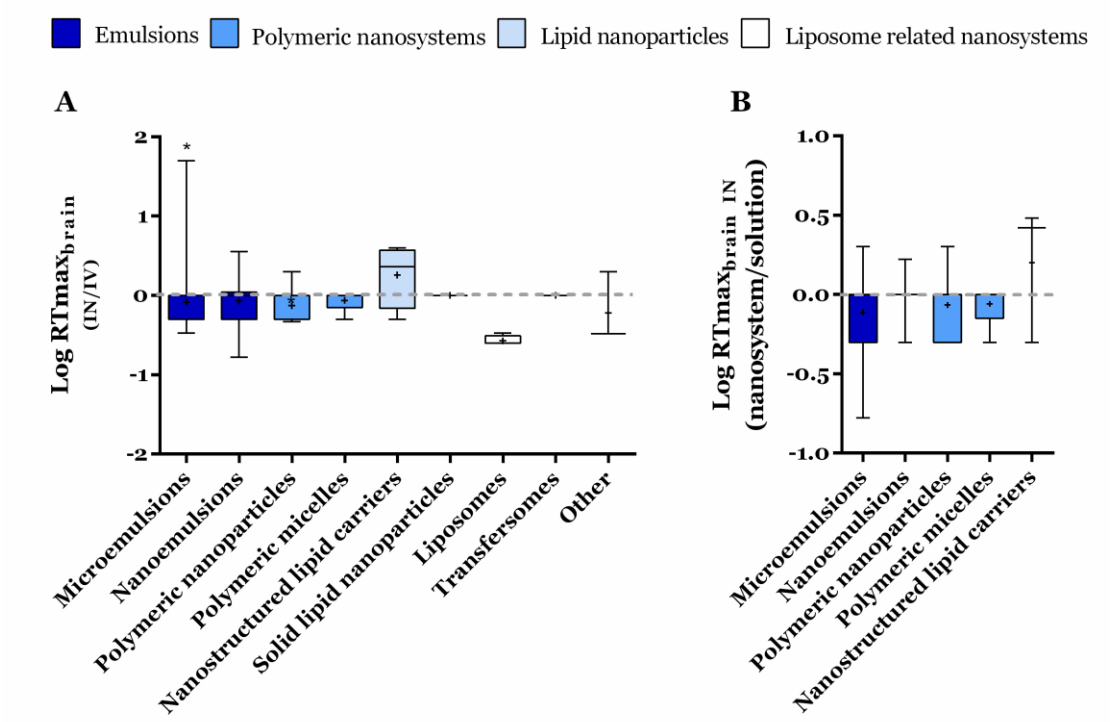

Figure 2.8. Representation of the Log of IN/IV (A) and nanosystem/solution (B) ratios of Tmax brain values for all nanosystem groups. Data correspond to median ± inter-quartile interval and range (box-plot) plus mean indicated by a small "plus" sign (+). Statistical significance of differences between means and reference "no-change" values by one-sample *t*-test when in presence of normal distribution, Wilcoxon signed-rank test when not (medians), * $p < 0.05$; IN – intranasal; IV – intravenous; RTmax – quotient of the time required to reach maximum brain concentration between intranasal and intravenous administrations, or between intranasal administration of the nanosystem and the respective solution.

2.4. Conclusions and final remarks

Pre-clinical brain targeting studies have generally put in evidence some of the advantages of intranasal brain drug delivery and have showed utility in comparing different carrier nanosystems, among themselves and with drug solutions. However, this review showed that there is a high heterogeneity on how these assays have been conducted, analyzed and reported in the scientific literature.

A greater uniformity in future reports of intranasal brain targeting studies would help with the interpretation of the potential value of newly developed formulations. The knowledge acquired from making this systematic review has led us to define some recommendations: a plain drug solution should be used intravenously instead or in addition to the developed nanosystem whenever possible, since the nanosystem itself can markedly alter the intrinsic pharmacokinetics and biodistribution of the drug; the validation parameters of the analytical methods should be described; pharmacokinetic ratios calculation (formulas and definition terms) should always be reported; when comparing different formulations, DTE% and $B\%_{\text{brain IN/IV}}$ should preferably be compared

after logarithmic transformation; the animal model could be harder to standardize, due to variable resources and administration techniques, but given the elevated number of animals required to characterize drug AUC in the brain, the use of mice, and a minimum study duration of 8 hours, might be good compromises.

Regarding the characterization of the formulations, in order to promote the progressive understanding of the factors that influence brain targeting, it should be as complete as possible. Formulations' characterization should include the nanosystem itself (mean hydrodynamic size, PDI, and zeta potential, detailing the conditions of its determination), and the final preparation (osmolality, pH, and viscosity, including temperature and velocity dependence), given the relevance of all these factors in nasal delivery. Drug release kinetics and the interaction with cells in the nasal mucosa are other important factors that can be informative, and further work to promote *in vitro* tests standardization is still required.

Nevertheless, the existing non-clinical brain targeting studies regarding the intranasal delivery of small drugs, although likely naturally biased for success cases, confirmed the expectation regarding the advantage of the intranasal route and the use of carrier nanosystems. Almost all (reported) nanosystems had favorable targeting ratios, and these were higher than the comparative intranasal drug solution. Moreover, success has already been obtained for a large group of drugs.

Regarding nanosystem classes, microemulsions (the most represented class, with the lowest mean particle size and lowest value range) and polymeric nanoparticles (the second most represented, with the highest mean particle size and highest value range) were only significantly different regarding Log $B\%_{brain\ IN/IV}$, which was higher for polymeric nanoparticles, as it was for polymeric micelles and nanostructured lipid carriers (also in comparison with microemulsions). These differences were lost when considering the comparison of nanosystems with the respective intranasal drug solution, especially in the case of polymeric micelles, where the number of reports is still small. This happened because some drugs reached the brain so efficiently, even as drug solutions, that further benefit from nanosystems became less evident. That being said, it was not possible, from the current global analysis, to clearly discriminate the overall superiority of a nanosystem class in relation to another with respect to brain targeting and bioavailability. We are only able to conclude that, in the reported works, nanosystems seem to be better than the respective drug solutions, particularity regarding brain bioavailability.

It is important to mention that, given the restrictive nature of the applied inclusion/exclusion criteria, it is not possible to generalize our results. In fact, only low molecular weight entities were included (protein and gene derived drugs were left out). Moreover, some nanosystem classes are underrepresented or have not been represented at all, because many studies describing intranasal nanosystem development do not design *in vivo* studies for DTE% or DTP% calculation.

Other factors, such as drug and nanosystem properties, are variables that might strongly influence the relative efficacy of a nanosystem. Furthermore, bias could derive from substantial differences in study design. Study duration, intravenous formulation, animal model, analytical method and drug strength are all factors that could influence the results in a great extent. In any case, we, as others have before us [14], would like to recognize that the extrapolation of results from animal models to humans carries a risk, since anatomy and physiology and, consequently, nanosystem performance is likely to be considerably different. Moreover, performance in both animals and humans might also be influenced by the delivery device itself, a variable that is mostly overlooked [19].

Chapter 3 – Intranasal fosphenytoin: the promise of phosphate esters in nose-to-brain delivery of poorly soluble drugs

3.1. Chapter overview and main objectives

With phenytoin being a low solubility antiepileptic, we hypothesized that using its hydrophilic prodrug, fosphenytoin, could be a viable approach for an intranasal formulation. Even if it is unlikely for fosphenytoin to undergo free passive absorption due to its anionic nature, it can be converted to phenytoin by phosphatases in the nasal cavity, as has been reported by Antunes Viegas *et al.* [21]. Our rationale was that the substantially increased drug strength, while formulating with safe excipients, and the local metabolization to the active diffusible form, could compensate for the reduced prodrug diffusion. Furthermore, by choosing a drug/prodrug pair already available in the market, this work aimed to serve as proof-of-concept that phosphate esters can be a useful strategy for nasal formulation development, to overcome poor bioavailability of many other poorly soluble drugs. Moreover, a few of the works included in the systematic review shown in Chapter 2 reported that some intranasal drug solutions already had quite good brain targeting efficacy, and in a few cases differences in brain targeting between the drug solution and the nanosystem were not statistically significant.

Drug solutions also have the advantage of requiring very simple preparation techniques. In order to increase the formulation's retention time in the nasal cavity, consequently allowing more time for drug absorption to occur and potentially increasing brain bioavailability, we considered two strategies: adding a mucoadhesive polymer – hydroxypropyl methylcellulose (HPMC); and/or adding a thermosensitive polymer – Poloxamer 407 (Pluronic® F-127, from now on referred to as Pluronic only) – which when heated can undergo sol-gel phase transition, if in solution at sufficient concentration. Both polymers have been previously used in nasal formulations' composition, alone or in combination [23,24]. The addition of albumin to the formulation was also evaluated, since it strongly binds to fosphenytoin and has been described to be actively transported from the nasal cavity to the brain [128]. The developed mucoadhesive and/or thermosensitive formulations of fosphenytoin were characterized regarding viscosity, osmolality, pH and *in vitro* drug release profile. The selected formulations were

then administered to mice in an *in vivo* pharmacokinetic study, to compare and characterize their pharmacokinetic profile.

3.2. Materials and methods

3.2.1. Materials

Part of fosphenytoin disodium (USP) was a gift sample from JPN Pharma (Mumbai, India), and another part was purchased from Jai Radhe Sales (Ahmedabad, India). Although it was provided as a hydrated disodium salt, mass concentration in the text will be indicated as calculated for the anhydrous acid form. Fosphenytoin and phenytoin (USP) reference standards and ketoprofen were acquired from Sigma-Aldrich (Steinheim, Germany), as were Pluronic, monobasic sodium phosphate and bovine serum albumin. Pentobarbital sodium injection solution (Eutasil®) was purchased from Ceva (Libourne, France). HPMC 2910 (USP) was bought from Acofarma (Barcelona, Spain). High-performance liquid chromatography (HPLC) grade methanol, analytical grade triethylamine, perchloric acid 70% (v/v) and diethyl ether, and sodium chloride and sodium hydrogen carbonate were all acquired from Fisher Scientific (Leicestershire, United Kingdom). Sodium acetate was bought from Merck (Darmstadt, Germany), potassium chloride from Chem-Lab (Zedelgem, Belgium), and dibasic sodium phosphate from Acros Organics (Geel, Belgium). Magnesium chloride and sodium hydroxide were purchased from Labkem (Barcelona, Spain). Calcium chloride and ortho-phosphoric acid 85% (v/v) were acquired from Panreac (Barcelona, Spain). Hydrochloric acid 37% (v/v) was bought from Fluka (Seelze, Germany). Water was always of ultra-pure grade (Milli-Q water apparatus, 0.22 μm filter, Merck, Darmstadt, Germany).

3.2.2. Formulation preparation

Formulations were prepared by weighing together all the necessary components: Pluronic and albumin were added in powder form; HPMC was added as a 2% (w/w) aqueous solution; and fosphenytoin was added either as a more concentrated aqueous solution, for preliminary batches with lower drug strengths, or as a powder, for final formulations with higher drug strengths. The pH was adjusted to 6 - 7 (nasal pH) for all formulations (Orion Star A211 pH meter, Thermo Fisher Scientific, Indonesia) and was then verified using universal indicator paper (Nahita, Auxilab S.L., Navarra, Spain). Water was also added by measuring the required mass, and formulations'

homogenization was achieved with mechanical or magnetic steering, at 4 ºC for preparations containing Pluronic and at room temperature for all others. For simplification purposes, percentual w/w concentrations [% (w/w)] will be indicated throughout the text as percentage only (%).

3.2.3. Rheology and osmolality

Viscosity measurements were made with a cone-plate rheometer (DV3T, Brookfield Ametek, Massachusetts, USA). Sample volume was 0.5 mL, and one of two spindles was selected – CP40Z or CP52Z. Temperature was regulated and maintained using a thermostated water bath (MultiTemp III Thermostatic Circulator, Thermo Fisher Scientific, New Hampshire, USA). Viscosity was measured at a constant temperature (20 ºC, mean room temperature, or 32 ºC, mean nasal cavity temperature) and varying shear rates. In the present work, we chose to determine and report zero shear viscosity, 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. For Newtonian fluids, zero shear viscosity was considered to be the value measured at the highest rotational speed (within the apparatus measurement range), for lower associated measurement error. Gelation was evaluated at a constant shear rate (100 s^{-1}) and varying temperatures. Each batch was measured only once, and values that were not within the torque interval correspondent to a minimum of 95% measurement accuracy were not considered.

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

3.2.4. *In vitro* drug release

In vitro drug release studies were performed using horizontal Ussing Chambers (Harvard Apparatus, NaviCyte, Hugstetten, Germany). Temperature was kept at 32 ºC (measured inside the chamber) using a thermostated water bath (Grant Instruments, Cambridge, England), and the membranes used in the assay were made of hydrophilic polyethersulfone, with a 0.2 μm pore size (Supor® membrane disc filters, Pall Life Sciences, Michigan, USA).

The bottom chamber was filled with 1.8 mL of nasal fluid simulant buffer, pH 6.5, 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) [129–131]. After the chambers were fully assembled, 200 µL of this same buffer were placed on the upper side of the membrane. After reaching the intended temperature, the buffer on the upper side of the membrane was replaced with 200 µL of the formulation. Homogenization of the bottom chamber fluid was achieved through magnetic steering (Micro Stirring Bars, 2 mm, VWR, United Kingdom). Samples of 100 µL were taken from the receiver chamber at 5, 10, 20, 40, 60, 80, 100, 120, 140, 160 and 180 minutes, and the volume was replaced with new buffer solution at every time point. Subsequently, drug quantification in the formulation and in the collected samples was done by spectrophotometry or HPLC, as described in the following sections 3.2.4.1. and 3.2.4.2. A simple fosphenytoin aqueous solution was used as positive control.

3.2.4.1. Spectrophotometric assay

Spectrophotometric assay selectivity was assessed by measuring vehicle, matrix and empty ultra-violet microplates (Greiner Bio-One, Germany) absorbance at 210 nm in a microplate spectrophotometer (xMark, Bio-Rad, Japan). Both the matrix (nasal fluid simulant buffer) and the empty wells had a relevant absorbance at the chosen wavelength, and thus the corresponding values were subtracted from the ones obtained for sample quantification, during data analysis. Vehicle interference was assessed by measuring the absorbance of the highest of the chosen polymer concentrations: Pluronic at 15% and HPMC at 0.5% (properly diluted).

Before absorbance reading, samples collected from the Ussing chambers (except the ones belonging to formulations containing albumin) were diluted 20-fold in nasal fluid simulant buffer. For the quantification of initial drug concentration, a sample was taken directly from the preparations and diluted 800-fold.

Method validation followed the Food and Drug Administration (FDA) guideline [132], for evaluation of the method's limit of quantification, linearity, precision, accuracy and selectivity for the analyte (fosphenytoin). Further detailed information can be found in the supplementary data (section S1.1.1).

3.2.4.2. High-performance liquid chromatography assay

In vitro drug release test samples belonging to formulations containing albumin were quantified by HPLC. The method was adapted from the one developed by Antunes Viegas *et al.* [21]. To obtain drug levels within the range of the calibration curves, samples collected from the Ussing chambers during the drug release assay were diluted 200-fold in nasal fluid simulant buffer, and samples taken directly from the formulations used in the assay were diluted 5000-fold. Perchloric acid at 10% (v/v) was then added in order to precipitate the albumin that was part of the formulation's composition.

Chromatographic apparatus consisted of a HPLC system (LC-2010A HT Liquid Chromatography) coupled with a diode-array detector (SPD-M20A), controlled automatically by the data acquisition software (LabSolutions, version 5.52), from Shimadzu (Kyoto, Japan). Analyte separation was performed at 30 ºC on a reversed-phase column (C18, 3 µm particle size, 55 × 4 mm) protected by a reversed-phase guard column (C18, 5 µm particle size, 4 × 4 mm), LiChroCART® Purospher® STAR models, both purchased from Merck (Darmstadt, Germany). Elution was done at 1 mL/min in isocratic mode, and the mobile phase was composed of (v:v) 36% methanol and 64% sodium phosphate buffer, 10 mM, pH 3, with 0.25% triethylamine, filtered (Nylaflo membrane, 0.2 µm pore size, Pall, USA) and degassed for 30 minutes (Branson Bransonic® M Mechanical Bath 5800, Missouri, USA) prior to injection. Sample injection volume was 20 µL. Analyte detection was done at 215 nm, with 20 minute runs.

Method validation concerning limit of quantification, linearity, precision, accuracy, selectivity and recovery of fosphenytoin followed the FDA guideline criteria [132]. Method selectivity was also evaluated for the formulation vehicle. Further detailed information can be found in the supplementary data (section 1.1.2).

3.2.5. *In vivo* pharmacokinetic study

3.2.5.1. Animal experimentation

In the animal experimentation studies we used adult male CD-1 mice, age ranging between 7 and 11 weeks, and weighing between 28 and 42 g. These animals came from our own institution's certified animal facility, and they were housed under controlled environmental conditions (12 hours light/dark cycle, 20 ± 2 ºC, 50 ± 5% relative humidity) with free access to tap water and standard rodent diet (4RF21, Mucedola, Italy). All animal procedures, including those to obtain blank matrices for validation experiments, were performed in conformity with the regulations of the European

Directive 2010/63/EU, regarding the protection of laboratory animals used for scientific purposes, and approved by the Local Animal Ethics Committee and by the competent national authority [Portuguese National Authority for Animal Health, Phytosanitation and Food Safety (DGAV – Direção Geral de Alimentação e Veterinária)].

A total of 176 animals were randomly divided into 4 experimental groups (11 time points, 4 mice per time point). Prior to formulation administration each mouse was anesthetized with a dose of 60 mg/kg of pentobarbital, through intraperitoneal injection. All formulations had a target fosphenytoin strength of 50 mg/g. A first group was given a slow intravenous tail-vein injection (over approximately 1 minute) of a fosphenytoin solution diluted 20-fold in physiological saline solution (sodium chloride 0.9%); a second group received a fosphenytoin solution intranasally; a third group received a fosphenytoin solution in HPMC at 0.5% (H0.5FOS) intranasally; and a fourth group was given a fosphenytoin solution in HPMC at 0.5% plus albumin at 2% (H0.5FOS + albumin) also intranasally. For intranasal administrations the mouse's body was laid on its left side, on top of a heating pad (plus a DC Temperature Controller 40-90-8D, FHC, Maine, USA). A flexible catheter, attached to a 50 µL syringe (Hamilton, Nevada, USA), was then inserted 3 to 4 mm into the right nostril. A volume of 5 µL per 30 g of body weight was administered once. After drug administration the mice were left to recover from anesthesia in a supine position, in a temperature-controlled environment.

3.2.5.2. *In vivo* sample collection, processing and high-performance liquid chromatography assay

After euthanasia at specific time points – 5, 10, 15, 30, 60, 120, 240, 360, 480, 720 and 1440 minutes – mice blood and brain were collected. Blood was collected to tubes containing ethylenediaminetetraacetic acid (1 mL capacity, with K3 EDTA, FL Medical, Italy), and after mild agitation 300 µL were transferred to an eppendorf tube already containing 300 µL of orthophosphoric acid 85% (v/v), making a blood:acid mixture in a 1:1 (v/v) ratio. The mixture was then kept on ice. Whole brains were homogenized (Ika Ultra-Turrax® T25 Basic, Staufen, Germany) in a mixture of water and orthophosphoric acid also in a 1:1 (v/v) ratio (1 g of tissue per 4 mL of mixture), and were likewise kept on ice. Afterwards, brain homogenates were centrifuged (MIKRO 200R microcentrifuge, Hettich, Tuttlingen, Germany) at 14000 rpm, 4 ºC, for 10 minutes. Both acidified blood and acidified brain homogenates' supernatants were stored at -20 ºC (RZ80FHRS freezer, Samsung, Seoul, South Korea) until needed. The purpose of the addition of

orthophosphoric acid to the tissues was to prevent fosphenytoin conversion to phenytoin.

During processing all samples were kept on ice. Initially, 20 µL of ketoprofen (the internal standard) spiking solution were added to 100 µL of brain homogenate supernatant sample or 200 µL of blood sample (either a blank matrix plus spiking solution, or a direct sample from the *in vivo* pharmacokinetic study). This was followed by liquid-liquid extraction, with 1000 µL of diethyl ether being added to each sample, which was subsequently vortexed for 30 seconds and then centrifuged (microcentrifuge, Gyrozen, Daejeon, South Korea) for 5 minutes, at 13500 rpm, at room temperature. The resulting organic phase was transferred to a glass tube, and the aqueous phase was then reextracted twice more, under the same conditions, with the combined organic phases being evaporated to dryness under a gas stream at 45 °C, and then reconstituted with 100 µL of mobile phase.

The chromatographic apparatus and analyte separation conditions were the same as for the quantification of the samples from the drug release study (section 2.4.2), but mobile phase was changed to 36% methanol and 64% sodium acetate buffer (10 mM, pH 5, with 0.25% triethylamine). Furthermore, fosphenytoin and phenytoin detection was done at 215 nm, but the detection of the internal standard was done at 280 nm. Run time and injection volume remained the same (20 minutes and 20 µL, respectively).

Method validation followed the FDA guideline as well [132], determining the same parameters as before, but now for both fosphenytoin and phenytoin (derived from the *in vivo* bioconversion of fosphenytoin). Further detailed information can be found in the supplementary data (section 1.1.3).

3.2.6. Data analysis

Statistical data analysis and graphical representation was done using the GraphPad Prism software, version 6.0. The significance level was set at 0.05.

Zero shear viscosity of non-Newtonian pseudoplastic fluids was estimated by fitting a non-linear regression model (*one phase decay*) to the "viscosity vs shear rate" data and determining the zero of the function (Y when X = 0), with or without prior variable transformation (X = Log_{10} X for Pluronic + HPMC formulations at 32 ºC). The half-gelation temperature ($T_{gel}50$) was considered to be the temperature at which viscosity is

at 50% of the correspondent to complete gelation, and was determined by applying a non-linear regression model [*log(agonist) vs. response, variable slope, four parameters*] to the "viscosity vs temperature" data.

The determination of the drug release parameters was done taking into account initial drug strength. Drug release rates were calculated using the Higuchi model [133,134], in which both time (X) and drug release percentage (Y) were transformed: the square root of X was calculated ($X = \sqrt{X}$), and Y was divided by the area of the membrane used in the assay ($Y = Y/0.64$). Then after these transformations a linear regression was applied, using mean values for each time point, and late time points for which correspondent values fell out of the linear zone were excluded. To assess whether they differed significantly between formulations, the drug release rates (slopes) were compared two-by-two using an F-test.

The existence of a correlation between total drug release percentage and zero shear viscosity at 32 ºC was assessed by using a Spearman's correlation test, two-tailed.

Differences between formulations' drug concentration levels in blood and brain were determined by two-way ANOVA analysis with Tukey's multiple comparisons post-test.

Pharmacokinetic parameters' determination was done using the add-in program for Microsoft Excel "PKSolver", a useful and reliable tool with results satisfactorily comparable to those of WinNonlin (the Pharmaceutical Industry's go-to tool) [135]. A non-compartmental analysis was done for all data, and all administrations were considered to be extravascular, even in the case of the intravenous group, since the administration was done at a slow rate. Maximum drug concentration (Cmax) and Tmax, in both blood and brain, were directly derived from the experimental data. $AUC_{o\text{-}t}$ was calculated through the linear trapezoidal method. $AUC_{o\text{-}inf}$ was calculated by adding $AUC_{o\text{-}t}$ to the last quantifiable drug concentration (with adequate precision and accuracy, C_{last}) divided by the elimination rate constant (k_{el}), with the formula being $AUC_{o\text{-}inf} = AUC_{o\text{-}t} + C_{last}/k_{el}$. k_{el} was estimated by applying a log-linear regression to the terminal segment of the "drug concentration vs time" curve. The elimination half-life ($t_{1/2el}$) was calculated by dividing ln2 by k_{el} (the formula being $t_{1/2el} = ln2/k_{el}$), and the mean residence time (MRT) by dividing the area under the first moment curve (AUMC) by the $AUC_{o\text{-}inf}$ (the formula being $MRT = AUMC/ AUC_{o\text{-}inf}$). The percentage of the AUC that was extrapolated, from the last quantifiable drug concentration to infinity, was also calculated [AUC_{extrap} (%)].

Absolute blood bioavailability ($B\%_{\text{blood IN/IV}}$) of the intranasal formulations was calculated with equation 3.1:

$$B\%_{blood\ IN/IV} = \frac{AUC_{\ blood_{IN}}}{AUC_{\ blood_{IV}}} \times 100 \qquad (3.1).$$

Relative blood bioavailability ($RB\%_{\text{blood}}$) was used to compare intranasally administered formulations to a simple intranasal aqueous drug solution, and it was calculated by equation 3.2:

$$RB\%_{blood} = \frac{(AUC_{\ blood_{IN}})_{formulation}}{(AUC_{\ blood_{IN}})_{solution}} \times 100 \qquad (3.2).$$

3.3. Results and discussion

3.3.1. Formulation development with rheology and osmolality characterization

Osmolality measurements and rheological studies were used to support the decision making regarding which polymer concentrations should be used in vehicle composition. Pluronic alone had an osmolality that ranged from around 130 to 260 mOsmol/kg, therefore being almost isotonic at the highest concentration (16%) (Table 3.1). This fact limits the amount of drug that can be dissolved in these vehicles without compromising the osmotic safety of the preparations. Oppositely, HPMC alone did not contribute measurably to the osmolality of the formulations. Consequently, mixed vehicles' osmolality values were similar to those of Pluronic alone.

Table 3.1. Viscosity and osmolality of vehicles containing HPMC only, Pluronic only, or mixtures of HPMC and Pluronic. Data correspond to 1 representative batch for each different vehicle. Osmolality data are presented as mean ± SEM. Viscosity was measured at 20 ºC, and in Newtonian fluids corresponds to the value at the highest torque. Viscosity of non-Newtonian fluids is represented as zero shear viscosity, inferred by non-linear regression analysis, and is presented as mean ± SEM.

Composition (w/w %)	**Viscosity at 20 ºC (cP)**	**R^2**	**n**	**Osmolality (mOsmol/kg)**
HPMC 0.2%	4.54 ± 0.05	0.9751	10	0
HPMC 0.5%	25.24 ± 0.15	0.9971	16	0
HPMC 1%	228.00 ± 1.04	0.9980	12	0
Pluronic 12%	10.11	-	-	132.2 ± 0.5
Pluronic 13%	12.61	-	-	165.0 ± 2.7
Pluronic 14%	16.12	-	-	196.2 ± 3.0
Pluronic 15%	22.38	-	-	222.2 ± 3.9
Pluronic 16%	29.04	-	-	260.8 ± 1.7
Pluronic 12% + HPMC 0.2%	13.54	-	-	135.7 ± 1.2
Pluronic 13% + HPMC 0.2%	15.71	-	-	160.0 ± 1.0
Pluronic 14% + HPMC 0.2%	19.84	-	-	204.0 ± 1.7
Pluronic 15% + HPMC 0.2%	26.75	-	-	245.4 ± 2.4
Pluronic 16% + HPMC 0.2%	41.97 ± 0.55	0.9455	13	285.8 ± 2.4

n – number of points (number of different speeds, one measurement per speed); HPMC – hydroxypropyl methylcellulose; R^2 – linear regression's coefficient of determination; SEM – standard error of the mean.

The rheological behavior of the vehicles over a range of shear rates, their zero shear viscosity and their gelation temperatures (when applicable) were then assessed, first for each polymer separately, and then in combination. At 20 ºC, HPMC showed non-Newtonian pseudoplastic behavior and Pluronic presented Newtonian behavior, for concentrations between 0.2 - 1% and 12 - 16%, respectively (not shown). Viscosity increased with increasing polymer concentration. For pseudoplastic fluids (HPMC dispersions), zero shear viscosity (inferred from regression analysis) was used to compare with Pluronic formulations' viscosity (Table 3.1).

As for the combination of the two polymers, it was only possible to obtain physically stable vehicles with HPMC at 0.2%, since with HPMC at 0.5 or 1% phase separation occurred after some time (varying between a few hours to a few days, sooner for higher polymer concentrations), and for the highest polymer concentrations a precipitate appeared. This physical instability in vehicles containing Pluronic and HPMC in combination (at higher polymer concentrations) has not, to the best of our knowledge, been previously reported in the scientific literature, even if a wide variety of studies have used them. Hence, we decided on combining Pluronic (at various concentrations) with

HPMC at 0.2% only. The addition of HPMC to Pluronic at 16% changed its rheological behavior from Newtonian to non-Newtonian (pseudoplastic) at 20 ºC (not shown).

Pluronic undergoes temperature induced sol-gel transitions. If the polymer's concentration is not high enough, it transitions from a low viscous fluid to a more viscous one, and not to a solid gel, but going forward we will still refer to it as gelation. With dispersions of Pluronic alone, gelation occurred at 15 and 16%, while for polymer concentrations equal or below 14% the viscosity only slightly increased with temperature increase up to 45 ºC (Figure 3.1A and supplementary data, section 1.2.1). The combination of the two polymers slightly increased the viscosity compared to Pluronic alone at 20 ºC (Table 3.1), but, more substantially, it also anticipated Pluronic's gelation (Figure 3.1A and supplementary data, section 1.2.1). Moreover, with Pluronic at 14% in combination with HPMC a transition to increased viscosity did in fact occur.

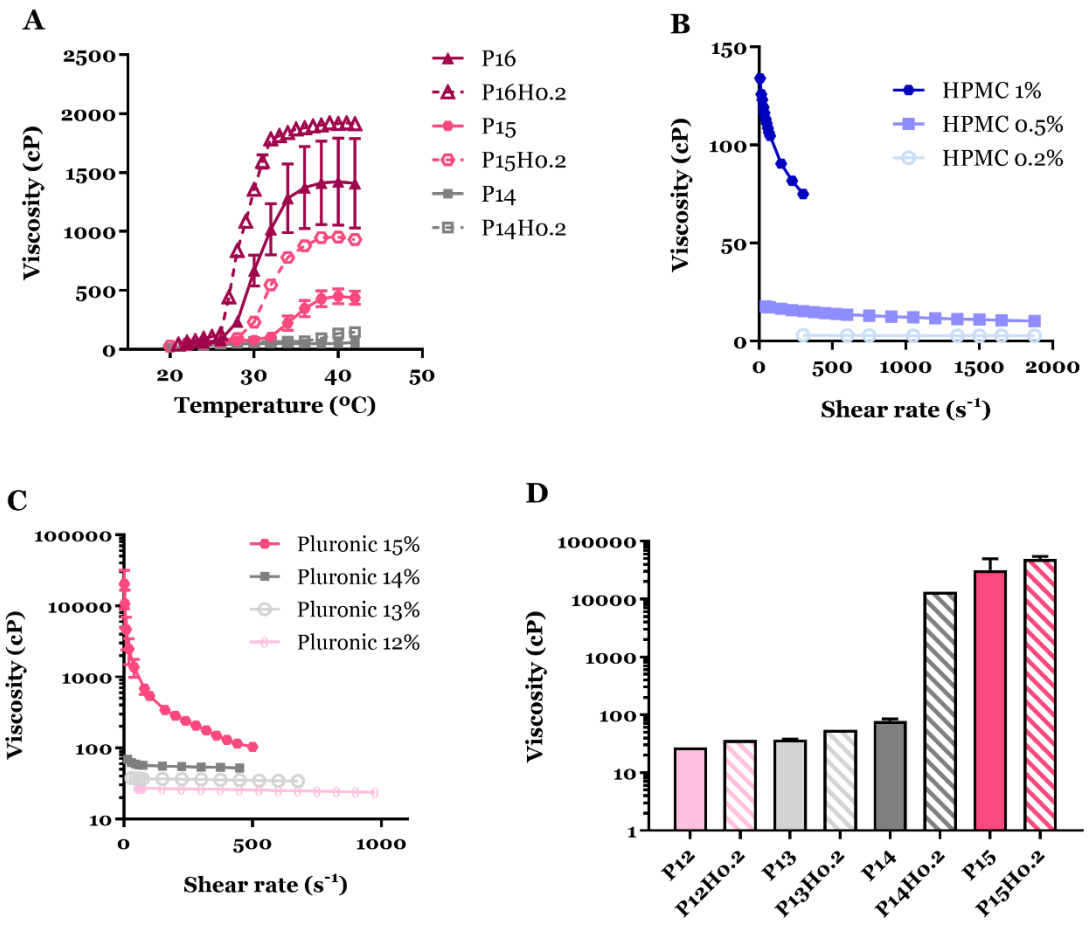

Figure 3.1. Viscosity variation with temperature increase at a constant shear rate (100 s^{-1}) for aqueous solutions containing Pluronic only (continuous connecting line) or Pluronic + HPMC (discontinuous connecting line) (A); evaluation of viscosity as function of the shear rate at 32 ºC of HPMC (B) or Pluronic (C) aqueous solutions; and zero shear viscosity at 32 ºC of aqueous solutions containing Pluronic only (clear pattern columns) or Pluronic + HPMC (striped pattern columns), determined by non-linear regression (D); 1 to 3 batches for each formulation. Data are presented as mean ± SEM. H or HPMC – hydroxypropyl methylcellulose; P – Pluronic; SEM – standard error of the mean.

At 32 ºC, both HPMC and Pluronic showed non-Newtonian pseudoplastic behavior (Figures 3.1B and 3.1C). For Pluronic at 16% it was not possible to evaluate viscosity over a wide shear velocity range, since at lower rotational speeds the torque was too high, with a corresponding viscosity above the spindle's measurement range. Furthermore, the addition of 0.2% HPMC to Pluronic resulted in increased zero shear viscosity at 32 ºC (Figure 3.1D).

In what concerns drug incorporation, for vehicles containing HPMC only the concentration of 1% was excluded because it led to a high zero shear viscosity at room temperature, which could hinder administration, especially through nasal instillation. Therefore, we selected the concentrations of 0.2 and 0.5%. Drug incorporation into these vehicles increased the zero shear viscosity at both studied temperatures, but not substantially (for the highest concentration, 0.5%, it only increased about 3 cP at 20 ºC and about 1 cP at 32 ºC) (Figure 3.2A and Tables 3.1 and 3.2). For vehicles containing Pluronic only, polymer concentration of 16% was excluded due to its gelation temperature being too low (T_{gel}50 30.4 ºC), having the risk of undergoing sol-gel transition at an increased room temperature (on a hot summer day, for example), which could also make it difficult to administer. Hence, concentrations from 12 to 15% were selected, and drug addition to these vehicles increased zero shear viscosity considerably, at both studied temperatures, but more substantially at 32 ºC (for the highest concentration, 15%, it increased about 26 cP at 20 ºC and about 122621 cP at 32 ºC) (Figure 3.2A and Tables 3.1 and 3.2). Moreover, there was also an effect of drug addition on gelation, which was anticipated, hence occurring at lower temperatures, with Pluronic at 15% plus fosphenytoin having a sol-gel transition near the mean nasal temperature (Figure 3.2B and supplementary data, section 1.2.1).

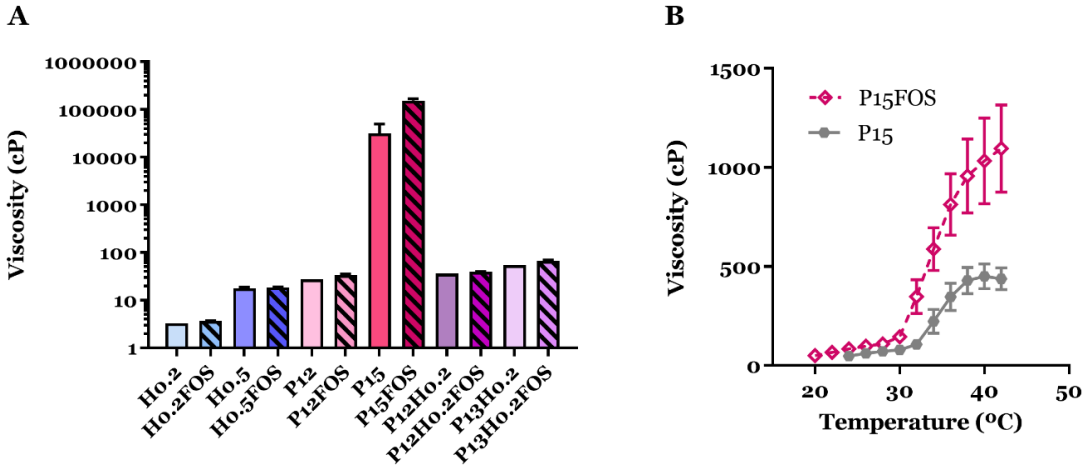

Figure 3.2. Zero shear viscosity at 32 ºC of vehicles (clear pattern columns) and drug formulations (striped pattern columns), determined by non-linear regression (A); and viscosity variation with temperature increase at a constant shear rate (100 s^{-1}) for Pluronic vehicle (continuous connecting line) and Pluronic drug formulation (discontinuous connecting line) (B); 1 to 3 batches for each formulation. Data are presented as mean ± SEM. FOS – fosphenytoin; H or HPMC – hydroxypropyl methylcellulose; P – Pluronic; SEM – standard error of the mean.

Table 3.2. Drug formulations' viscosity and zero shear viscosity, at 20 and 32 ºC, and osmolality. Three batches for each different formulation. Data are presented as mean ± SEM.

Formulation	Zero shear viscosity at 20 ºC (cP)	R^2	n	Zero shear viscosity at 32 ºC (cP)	R^2	n	Osmolality (mOsmol/kg)	Fosphenytoin strength (mg/g)
H0.2FOS	4.76 ± 0.01	0.9991	12	3.68 ± 0.01	0.9971	16	263.7 ± 14.9	29.27 ± 2.77
H0.5FOS	27.73 ± 0.15	0.9983	13	18.56 ± 0.06	0.9983	13	251.1 ± 14.9	26.64 ± 1.93
P12FOS	16.22 ± 0.39	-	-	33.51 ± 0.09	0.9882	16	475.3 ± 20.5	25.93 ± 3.32
P15FOS	48.54 ± 2.78	-	-	154133 ± 11645	0.9837	19	616.3 ± 26.0	27.53 ± 1.80
P12H0.2FOS	21.08 ± 1.11	-	-	39.35 ± 0.23	0.9586	13	502.5 ± 22.0	31.24 ± 5.25
P13H0.2FOS	28.80 ± 1.12	-	-	65.94 ± 0.77	0.9790	12	545.6 ± 19.7	30.15 ± 2.84

n – number of points (number of different speeds, one measurement per speed); FOS – fosphenytoin; H – hydroxypropyl methylcellulose; P – Pluronic; R^2 – linear regression's coefficient of determination; SEM – standard error of the mean.

As for mixed vehicles, drug incorporation was only possible for the two lowest Pluronic concentrations – 12 and 13% (plus HPMC at 0.2%), since for Pluronic at higher concentrations fosphenytoin had poor solubility, forming a drug precipitate. Drug addition to the selected polymer mixtures gave rise to an increased viscosity (for the highest concentrations, Pluronic at 13% and HPMC at 0.2%, it increased about 13 cP at 20 ºC and about 11 cP at 32 ºC) (Figure 3.2A and Tables 3.1 and 3.2).

Drug incorporation into the selected vehicles led to slightly hypotonic formulations for preparations containing HPMC only, and moderately hypertonic formulations for preparations containing Pluronic only or Pluronic + HPMC (Table 3.2). Although hypertonic, these last formulations were still within the established limits for marketed nasal preparations [136], at the current drug concentration range.

In addition to selecting the desired polymers and their concentrations, we sought to find a strategy that could potentially increase drug targeting to the brain, and one that was, ideally, relatively simple. Therefore, we chose adding albumin at 2% (w/w) to the selected preparations, as it has been described to be actively transported from the nasal cavity to the brain [128]. Nevertheless, for formulations containing just Pluronic, only polymer concentrations of 12% allowed the addition of this protein without compromising physical stability, with the preparations with Pluronic at higher concentrations acquiring a high turbidity. The same happened for the mixed vehicles, regardless of composition. On the contrary, formulations containing HPMC only, at both 0.2 and 0.5%, were physically stable. Furthermore, the addition of albumin to the vehicles containing Pluronic at 12% or HPMC at 0.2 or 0.5% did not noticeably alter their viscosity (data not shown).

3.3.2. *In vitro* drug release

The viscosity of a formulation may, on the one hand, increase its retention in the nasal cavity, consequently increasing bioavailability through this route, but on the other hand it can also considerably decrease drug diffusion and release rate, having a counterproductive effect. Therefore, to further assist on formulation selection, the *in vitro* drug release rates of the preparations that were selected during the rheological studies phase – H0.2FOS, H0.5FOS, P12FOS, P15FOS, P12H0.2FOS and P13H0.2FOS – were evaluated using horizontal Ussing chambers, and compared to a fosphenytoin aqueous solution (positive control).

Since HPMC and Pluronic at the highest concentrations (Pluronic at 15% and HPMC at 0.5%) did not interfere with drug absorption at 210 nm (at the dilution used in the assay), a simple spectrophotometric method was developed for fosphenytoin quantification. For the formulation H0.5FOS + albumin *in vitro* drug release assay sample quantification was done by HPLC, since albumin showed high absorbance at 210 nm, thereby interfering with drug quantification in the spectrophotometric method. Validation results for both assays are given in the supplementary data (sections 1.2.2 and 1.2.3).

In what concerns percentual drug release (Figure 3.3) and percentual drug release rate (Table 3.3) H0.2FOS and P12FOS were not significantly different from the drug solution or from each other, although H0.2FOS appeared to release fosphenytoin slightly faster than P12FOS. Drug release from H0.5FOS was as fast as from P12FOS, and they both reached a total drug release similar to H0.2FOS (at the final time point). P15FOS had a more sustained, significantly slower drug release than all other formulations, which was to be expected given its very high viscosity at 32 ºC (which was likely to reduce drug diffusion), except when compared to P12H0.2FOS and P13H0.2FOS, which despite having a much lower viscosity than P15FOS were the slowest in releasing drug over time, also releasing the least amount after 180 minutes (3 hours).

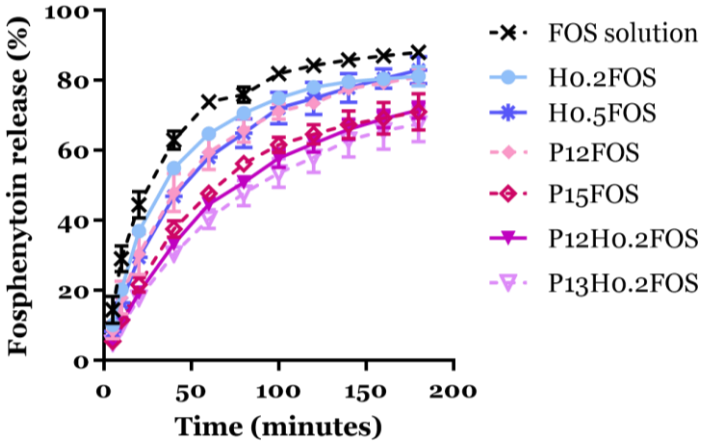

Figure 3.3. Fosphenytoin's percentual drug release between 5 and 180 minutes. FOS – fosphenytoin; H - hydroxypropyl methylcellulose; P – Pluronic.

Table 3.3. Fosphenytoin's percentual drug release rate, calculated by applying a linear regression to the plotting of the square root of time (X = √X) versus percentual drug release divided by the area (cm^2) of the membrane used in the assay (Y = Y/0.64). Significance matrix is shown for the difference between formulations (slopes' comparison using an F test).

Formulation	**Percentual drug release**		**Significance matrix**					
	R^2	**Drug release rate ($\%\cdot cm^{-2}\cdot min^{-1/2}$)**	**H0.2FOS**	**H0.5FOS**	**P12FOS**	**P15FOS**	**P12H0.2FOS**	**P13H0.2FOS**
FOS solution	0.9905	18.3 ± 1.3	NS	0.0190	NS	< 0.0001	< 0.0001	< 0.0001
H0.2FOS	0.9902	15.8 ± 0.9	-	< 0.0001	NS	< 0.0001	< 0.0001	< 0.0001
H0.5FOS	0.9986	14.3 ± 0.3	-	-	NS	0.0004	< 0.0001	< 0.0001
P12FOS	0.9988	14.5 ± 0.3	-	-	-	0.0260	0.0014	0.0008
P15FOS	0.9972	11.7 ± 0.3	-	-	-	-	0.0223	0.0036
P12H0.2FOS	0.9966	10.6 ± 0.3	-	-	-	-	-	NS
P13H0.2FOS	0.9981	9.9 ± 0.2	-	-	-	-	-	-

FOS – fosphenytoin; H - hydroxypropyl methylcellulose; NS – not significant (statistical difference); P – Pluronic; R^2 – linear regression's coefficient of determination.

Theoretically, a formulation with a higher viscosity slows down drug diffusion more, leading to a more sustained and/or overall lower release. Our results were mostly in agreement with that, with a strong negative correlation existing between zero shear viscosity at 32 ºC and drug release percentage at the final time point (Spearman's correlation test, two-tailed, $p < 0.0001$, $r_S = -0.756$). Nevertheless, the formulations combining both the thermosensitive and the mucoadhesive polymers, which were not most viscous, had the slowest drug release. This might be due to an interaction between the two polymers and the drug.

Considering osmolality, viscosity and *in vitro* drug release results, we decided that the formulation containing HPMC at 0.5% would be the best choice for further studies, due to several reasons: it was potentially mucoadhesive and had a high enough viscosity at 32 ºC to possibly help retain the formulation in the nasal cavity, but not so high that it slowed or decreased drug release in a substantial way; viscosity at 20 ºC allowed administration; and regarding osmolality (and osmotic safety) it allowed a higher drug strength than the preparations containing Pluronic.

Hence, from the formulations containing albumin we also chose HPMC at 0.5% and went on to verifying whether the addition of albumin to the polymeric preparation altered drug release in any way. Results showed that there was no considerable difference in drug release from H0.5FOS + albumin, in any of the studied parameters, being similar to those obtained for H0.5FOS (data not shown).

Formulations H0.5FOS and H0.5FOS + albumin were, therefore, selected for further evaluation in *in vivo* pharmacokinetic studies, for administration through the intranasal route. Drug strength was increased and set at 50 mg/g, corresponding to an osmolality between 300 and 400 mOsmol/kg, values that are regarded as safe for intranasal administration [136].

3.3.3. *In vivo* pharmacokinetic study

Bioanalytical method validation results are presented in the supplementary data (section 1.2.4).

Fosphenytoin is a dianionic molecule with reduced permeability, but in a previous *ex vivo* permeation study a small amount of this prodrug was shown to permeate [21]. We wished to know whether fosphenytoin could reach the brain in its unmetabolized form

when administered intranasally. Despite the developed procedure, preventing fosphenytoin conversion to phenytoin after sample collection, fosphenytoin levels were always below the limit of quantification of 0.3 μg/mL (blood) and 1.5 μg/g (brain), even at short time points (5 or 10 minutes). However, it is not certain whether *in vivo* the absence of fosphenytoin quantification is due to it being converted to phenytoin before absorption, or if some fosphenytoin may permeate as such and be converted immediately afterwards, since intravenous administration also led to unquantifiable fosphenytoin. In fact, fosphenytoin has a short and species-dependent conversion half-life. A few studies show that fosphenytoin's conversion to phenytoin in the blood is complete about 15 minutes after intravenous infusion in humans, and 10 minutes after intravenous infusion or intramuscular administration in rabbits, however in rats conversion is essentially complete after 5 minutes [137–139]. Moreover, all those studies used higher drug doses, varying between 10 and 30 mg of phenytoin equivalents per kg of animal body mass, whereas in our study drug doses only reached 5.8 mg/kg. Logically, with lower doses conversion is likely to be completed sooner.

Looking at phenytoin's brain and blood concentration over time (Figure 3.4), it is noticeable that the intravenous solution produced higher concentrations at earlier time points ($p < 0.01$, two-way ANOVA). Tmax in the brain was 120 minutes (2 hours) for the intravenous administration and 240 minutes (4 hours) for the intranasal administrations (Table 3.4). Even assuming that effective brain concentrations can be achieved before Tmax, it still indicates that the intranasal delivery of prodrugs like fosphenytoin, at least with the present formulation strategy, may not lead to a fast therapeutic effect, as is required in emergency situations. This is possibly explained by the need of prodrug conversion occurring before effective drug diffusion through the nasal mucosa.

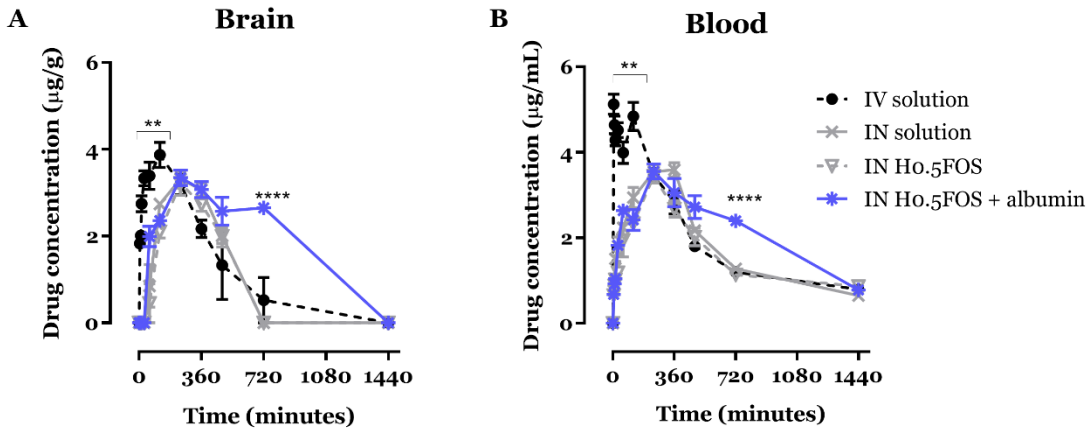

Figure 3.4. Curves of phenytoin concentration as a function of time in brain (A) and blood (B). Data are presented as mean ± SEM. Only significance levels for significant differences obtained when comparing one condition to all others are shown: **** $p < 0.0001$ and ** $p < 0.01$, two-way ANOVA analysis with Tukey's multiple comparisons post-test; FOS – fosphenytoin; H – hydroxypropyl methylcellulose; IN – intranasal; IV – intravenous.

Table 3.4. Pharmacokinetic parameters determined for phenytoin levels, in both brain and blood, for all tested formulations and administration routes.

Formulation	**Fosphenytoin solution**				**Ho.5FOS**		**Ho.5FOS + albumin**	
Administration route	**Intravenous**		**Intranasal**		**Intranasal**		**Intranasal**	
Matrix	**Brain**	**Blood**	**Brain**	**Blood**	**Brain**	**Blood**	**Brain**	**Blood**
Cmax (µg/g or µg/mL)	3.88	5.13	3.32	3.59	3.22	3.44	3.35	3.55
Tmax (min)	120	5	240	360	240	240	240	240
$t_{1/2el}$ (min)	176	503	332	584	348	624	1394	553
k_{el} (min^{-1})	0.0039	0.0014	0.0021	0.0012	0.0020	0.0011	0.0005	0.0013
MRT (min)	311	756	579	817	622	1003	2117	839
AUC_{0-t} (µg.min/g or µg.min/mL)	1570	2751	1185	2523	1049	2297	1885	3110
AUC_{0-inf} (µg.min/g or µg.min/mL)	1703	3325	2149	3069	2050	3076	7226	3734
AUC_{extrap} (%)	7.79	17.26	44.85	17.80	48.82	25.33	73.92	16.70
$B\%_{blood\ IN/IV}$	-		-	92	-	83	-	113
$RB\%_{blood}$	-		-	-	-	91	-	123

AUC_{0-t} – area under the "drug concentration vs time" curve, from time zero to the last quantifiable drug concentration; AUC_{0-inf} – area under the "drug concentration vs time" curve, from time zero to infinity; AUC_{extrap} (%) – percentage of the area under the "drug concentration vs time" curve that was extrapolated, from the last quantifiable drug concentration to infinity; $B\%_{blood\ IN/IV}$ – absolute blood bioavailability (intranasal formulation vs intravenous solution); Cmax – maximum drug concentration; FOS – fosphenytoin; H – hydroxypropyl methylcellulose; IN – intranasal; IV – intravenous; k_{el} – elimination rate constant; MRT – mean residence time; $RB\%_{blood}$ – relative blood bioavailability (intranasal formulation vs intranasal solution); $t_{1/2el}$ – elimination half-life; Tmax – time to reach maximum drug concentration.

The brain and blood profiles of the intranasal drug solution and the intranasal H0.5FOS formulation largely overlap (Figure 3.4). Therefore, overall HPMC did not benefit nor reduce drug absorption. Phenytoin's Cmax in the brain was relatively similar between all formulations and administration routes (3.2 to 3.9 μg/g) (Table 3.4). Blood Cmax was also quite similar for all intranasal formulations (3.4 to 3.6 μg/mL) but was substantially higher for the intravenous administration (5.1 μg/mL), which suggests that the intranasal route could be safer in what concerns maximum systemic drug levels. It is also important to mention that this small single dose administration already achieved half of the lower limit of these rodents' therapeutic level, which is around 7-12 μg/mL [140].

The addition of albumin at 2% to the formulation (H0.5FOS + albumin) prolonged phenytoin's blood and brain drug levels, since phenytoin's concentration at 480 minutes (8 hours) and, more significantly, at 720 minutes (12 hours) was clearly higher that the obtained with the other formulations ($p < 0.0001$, two-way ANOVA) (Figure 3.4). This could, hypothetically, be explained by intracellular neural transport to the brain, as previously demonstrated for albumin [128], since intracellular transport is slow when compared to extracellular diffusion, and/or increased retention in the nasal cavity.

Given the larger uncertainty obtained in phenytoin's pharmacokinetic profile in the brain after intravenous administration, partially due to the high limit of quantification of the method, elimination and AUC parameters are shown (Table 3.4) but are likely less reliable in this matrix. Therefore, we decided not to calculate absolute brain bioavailability or brain targeting ratios. However, blood bioavailability is for phenytoin a good indicator of brain bioavailability as well. All intranasal formulations, and especially the formulation with albumin, led to high absolute bioavailability, with the formulation containing albumin having a bioavailability about 20% higher than the simple aqueous intranasal fosphenytoin solution (Table 3.4). This is clearly associated with the sustained phenytoin levels.

Phenytoin is a good example of a very low water solubility drug, estimated as being only 0.032 mg/mL for its sodium salt [141]. Its marketed solution, to be administered through intravenous infusion, reaches a drug strength of 50 mg/mL, but drug solubilization is only achieved by using 40% propylene glycol and 10% ethanol, a high percentage of potentially harmful organic solvents, and pH 12, a high pH that can also be associated with toxicity. Serious and sometimes fatal dermatological reactions have been reported to occur, varying from mild irritation and inflammation to tissue necrosis [142]. It is, for sure, not suitable for nasal administration. A few studies report attempts to increase phenytoin's solubility without the use of potentially toxic excipients, however, achieved

drug concentrations have mostly been low. And even though for some therapeutic indications a low phenytoin strength (0.01 to 0.2 mg/mL) might be enough, and is even desired, such as in topical wound healing, for epilepsy treatment, especially for the management of seizures, higher strengths are required [143,144]. Self-emulsifying drug delivery systems for oral delivery reported phenytoin concentrations reaching up to 25 mg/mL [145,146]. In our study, by using phenytoin's hydrophilic prodrug, fosphenytoin, we obtained a higher strength of phenytoin equivalents than any of the reported works, reaching around 34.8 mg/mL (50 mg/mL of fosphenytoin). Furthermore, we could still further increase it by 2- or 3-fold, as osmolality was not very high yet (and fosphenytoin has a higher aqueous solubility than the concentration we selected) and/or perform multiple administrations. Thus, therapeutic levels are likely achievable. Moreover, the developed fosphenytoin formulations are simple to prepare and safe to administer, since they have no potentially toxic excipients in their composition.

3.4. Conclusion

In conclusion, intranasal fosphenytoin efficiently permeated and/or was converted to the diffusible active form *in vivo*, reaching high absolute bioavailability. Therefore, the use of phosphate ester prodrugs is an efficient and safe way of increasing the intranasal delivery of poorly soluble drugs such as phenytoin. Moreover, the addition of albumin to the formulation can prolong the drug's disposition in the brain compared to other intranasal formulations, enabling a better drug targeting.

Chapter 4 – Nanoemulsions and nanoemulgels of phenytoin and fosphenytoin

4.1. Chapter overview and main objectives

From the results presented in Chapter 2 it was not possible to clearly discriminate the overall superiority of a nanosystem class in relation to another in what concerns brain targeting and bioavailability. Therefore, the reasons behind the choice of which type of nanosystem formulation should be developed for phenytoin solubilization fell upon practical advantages. Hence, we chose to develop oil-in-water nanoemulsions, where the drug would be solubilized in the internal phase. These nanosystems can be quite easy to prepare, since certain formulas emulsify spontaneously just by adding the aqueous phase component to the mixture of oil and surfactants in the right proportions [19]. Furthermore, the results from the systematic review also showed that, in general, nanometric emulsions had good brain targeting efficacy.

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. As has been previously mentioned, Pluronic F-127 is a thermosensitive polymer, whose viscosity increases with temperature increase, having the ability to undergo sol-gel phase transition (if at sufficient concentration in solution). It has been extensively used in the development of *in situ* nasal gels, as have carbomers, which in association with poloxamers combine viscosity increase and mucoadhesive properties [23,147]. 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 the work shown in this chapter 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.

4.2. Materials and methods

4.2.1. Materials

The acid form of phenytoin used in formulation development was purchased from Acros Organics (Geel, Belgium), whilst the USP reference standard, for HPLC, was bought from Sigma-Aldrich (Steinheim, Germany). Fosphenytoin used in formulations was a gift sample from JPN Pharma (Mumbai, India), provided as a disodium 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 triethylamine, 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). Carbopol® 971P was donated by Lubrizol (Brussels, Belgium). Miglyol® 812, Tween 80 and Transcutol® P were all acquired from Acofarma® (Barcelona, Spain). Hydrochloric acid was bought from Fluka (Seelze, Germany). 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).

4.2.2. 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. 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 containing Pluronic and fosphenytoin, either way guarantying that the pH of all formulations was close to neutrality.

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, with mild manual or magnetic agitation at room temperature. The rest of the aqueous phase was then added to invert the emulsion, 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. It consisted of mechanical extrusion, at room temperature, using a mini-extruder set (Avanti Polar Lipids, Alabama, United States of America), through 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 4.1.

Table 4.1. Summary of the composition of the most relevant developed formulations.

	Final concentration in the preconcentrate (mg/g)				Aqueous phase		Fosphenytoin final concentration (mg/g)	Total drug strength (expressed as phenytoin, mg/g)
Formula name	**Miglyol 812**	**Tween 80**	**Transcutol**	**Phenytoin**	**Composition (w/w %, before emulsification)**	**% (w/w)**		
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_{P+FOS}	-	-	-	-	Pluronic (16%) + Fosphenytoin (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; TG – thermosensitive gel; TN – thermosensitive nanoemulgel.

4.2.3. 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 droplet mean size and 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 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.

4.2.4. 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 or 20 ºC). 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.

4.2.5. *In vitro* drug release assay

The *in vitro* drug release study was performed under similar conditions as described in Chapter 3 (section 3.2.4), using horizontal Ussing Chambers, with temperature being kept at 32 ºC and using membranes with a 0.2 μm pore size.

Again, the bottom chamber was filled with 1.8 mL of nasal fluid simulant buffer, pH 6.5, having the same composition as described in Chapter 3 (section 3.2.4). 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 would only 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 stirring.

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.

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. Chromatographic apparatus and conditions were the same as before (Chapter 3, section 3.2.4.2): reversed-phase column and guard column, elution at 1 mL/min, mobile phase made of 36% methanol and 64% aqueous phase (sodium phosphate buffer, 10 mM, pH 3, with 0.25% triethylamine), analyte detection at 215 nm for both fosphenytoin and phenytoin, 20 minute runs, injection volume of 20 µL. The method was validated following the Food and Drug Administration guideline criteria [132] (further information on validation methods and results in the supplementary data, sections 2.1.1 and 2.2.1, respectively).

4.2.6. 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.

The 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 between formulations' viscosity (zero

shear for non-Newtonian behavior) and between viscosity values 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.

$T_{gel}50$ was calculated 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 between formulations' mean size and PDI, and also to compare between a different number of extrusion cycles in the same formulation.

Similarly to the previous drug release assay, for the calculation of the drug release rate the Higuchi model was applied [133,134]. 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.

4.3. Results and discussion

4.3.1. Phenytoin nanoemulsion development

The work regarding the preliminary steps of phenytoin nanoemulsion development was done in the research group beforehand, in the context of a compounding formula for a phenytoin oral self-emulsifying drug delivery system (SEEDS). The lead formula, published by Atef and Belmonte [146], 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 a lower cosolvent amount than the used in the oral SEEDS was desired, and formulations should be isotonic to slightly hypertonic (within the established limits for marketed nasal preparations) [136]. After several tests with varying excipient proportions, it was established that, for maximum phenytoin solubilization in the formula, either Transcutol proportion should be > 0.25 or Tween 80 > 0.6 (Transcutol vs Tween 80 proportion). Furthermore, to avoid drug precipitation upon emulsification, Transcutol proportion should be within the range of approximately 0.2 to 0.35. Among the series of tested excipient proportions (screening tests, data not shown), one formula achieved good characteristics and maximum phenytoin solubilization - 20% oil, 46% surfactant and 34% cosolvent. This preconcentrate originated a nanoemulsion at 10% (w/w) in water, with 203.5 ± 4.6 nm mean size, PDI of 0.316 and an osmolality of 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 expected to permeate, not contributing to the preparation's tonicity.

Nevertheless, it was only possible to incorporate 10 mg/g of phenytoin in the preconcentrate without the occurrence of precipitation upon emulsification, 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. These formulations 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 [21]. In this study, while an initial dose of phenytoin was readily available for passive diffusion, in parallel more phenytoin was generated *in situ* due to the conversion of the prodrug to the active form, and some permeation of fosphenytoin by alternative pathways also occured.

The aqueous phase alone (fosphenytoin solution) was isotonic (283 mOsmol/kg). Nevertheless, the resulting emulsions were moderately to highly hyperosmotic (Table 4.2). However, as discussed above 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*.

Table 4.2. 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	694.7	216.4	0.305	-20.8	2.15	1.56
	SD	1.5	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	1611.2	209.2	0.263	-18.6	22.73	20.05
	SD	7.1	21.7	0.036	0.5	-	0.10
	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 that were characterized; PDI – polydispersity index; R^2 – linear regression's coefficient of determination; RMPS - replicate measurements per sample, contemplated 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 is to be expected from spontaneous emulsification (data not shown). If the preparations were 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 4.1. This time, characterization was done immediately after homogenization.

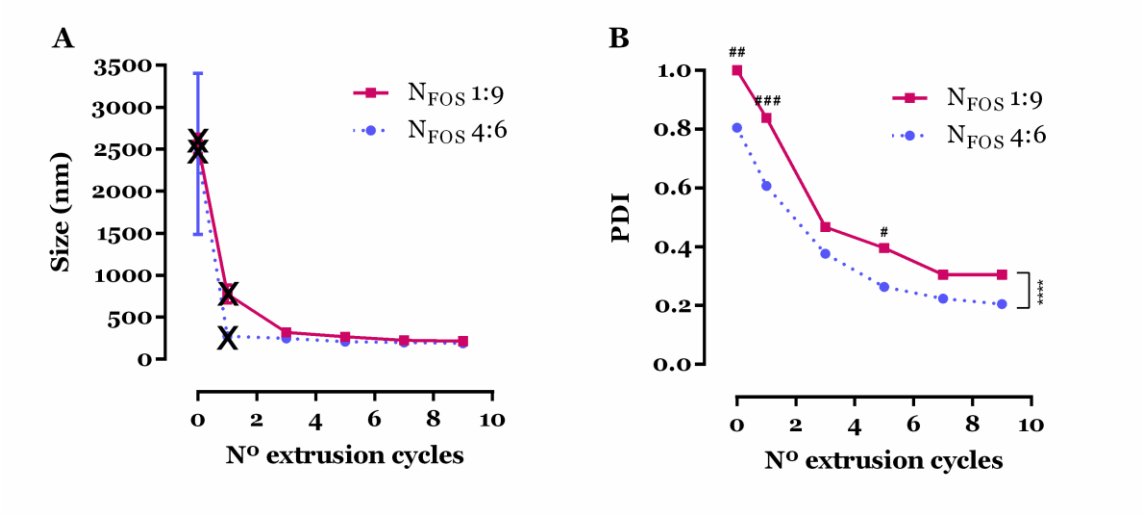

Figure 4.1. Premix membrane emulsification effect on droplet size dispersion of the N_{FOS} 1:9 and N_{FOS} 4:6 emulsions. Mean size (A) and PDI (B) dependence on the increasing number of extrusion cycles. Data are shown as mean ± SEM. Cross mark signals mean size results that showed poor quality, consequently 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 equal or 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 4.2.

At this stage, while drug strength was successfully increased, viscosity values were low (Table 4.2). This might be adequate for nasal administration in 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 of time, by reducing mucociliary clearance [14,19]. Hence, for extended drug release we sought to increase the preparation's viscosity.

4.3.2. Pluronic thermosensitive nanoemulgel development

Previous work done in the research group led to the development of a gelling emulsion (thermosensitive emulgel), from the former leading preconcentrate, and based on previously reported Pluronic/Carbopol thermosensitive gels [148]. After screening different Pluronic concentrations, the aqueous phase was set at 90%, containing 17% Pluronic (w/w), 0.2% Carbopol (w/w) and 22.3 mg/g of fosphenytoin (reduction of fosphenytoin's strength to not increase osmolality too much), which resulted in a drug strength equivalent to 15 mg/g of phenytoin (1.5%) in the final formulation. But although the produced thermosensitive emulgel was relatively stable, with a reasonable amount of drug, the gelation temperature was too low for nasal instillation (complete gelation occurring between 25 - 30 ºC), especially during the summer, requiring it to be refrigerated immediately prior to 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 this formulation could be beneficial, ensuring that a complete semisolid consistency is obtained shortly after spreading on the skin.

In order to produce formulations with a more desirable viscosity at room temperature, for intranasal instillation purposes, we decided to decrease Pluronic's 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) preconcentrate proportion. The preconcentrate contained 10 mg/g of phenytoin and the aqueous phase contained 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 4.2), suggesting heterogeneity, therefore we decided to perform premix membrane homogenization once more. 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 nanoemulgels.

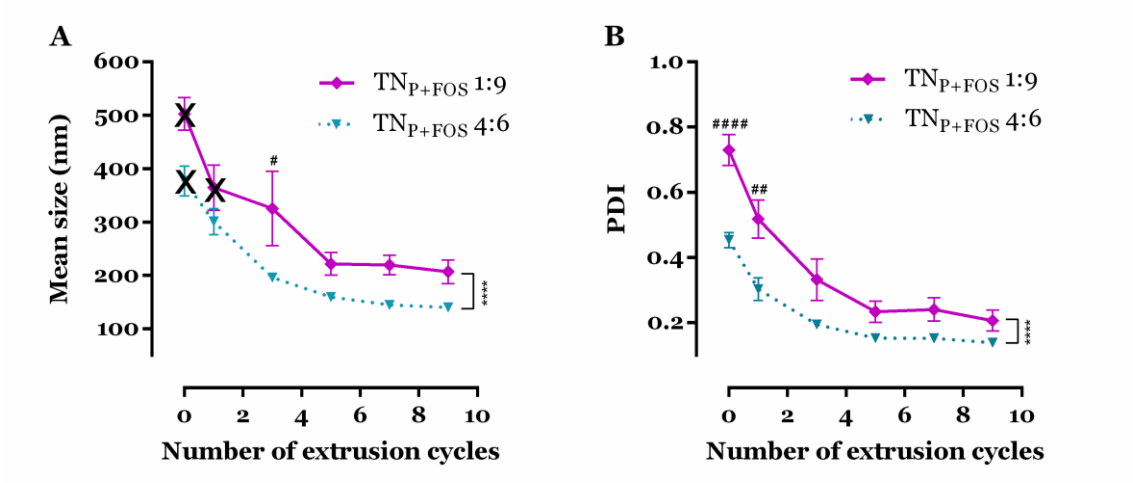

Figure 4.2. Premix membrane emulsification effect on droplet size dispersion of the TN_{P+FOS} 4:6 and TN_{P+FOS} 1:9 nanoemulgels. Mean size (A) and PDI (B) dependence on the increasing number of extrusion cycles. Data are shown as mean ± SEM. Cross mark signals mean size results that showed poor quality, 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; TN – thermosensitive nanoemulgel.

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 4.3).

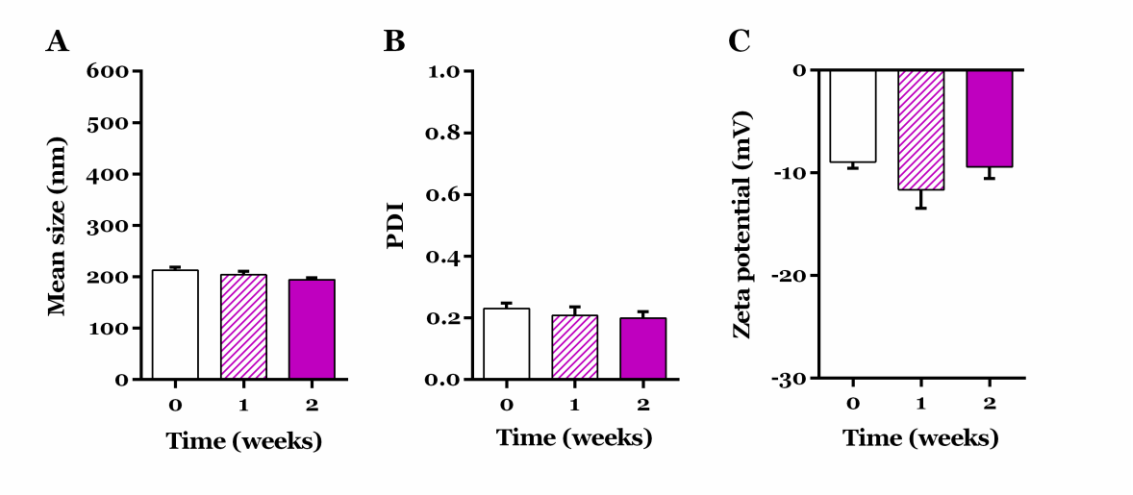

Figure 4.3. Droplet dispersion characterization in the nanoemulgel TN_{P+FOS} 1:9, over two weeks. Mean size (A), PDI (B) and zeta potential (C). Data are shown as mean ± SEM for 3 independent formulation batches. Mean size and PDI decreased with time ($p < 0.0001$ and $p < 0.05$, respectively, by one-way ANOVA with post-test for linear trend). FOS – fosphenytoin; PDI - polydispersity index; SEM - standard error of the mean; TN – thermosensitive nanoemulgel.

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, TN_{P+FOS} 4:6 was more viscous at 20 ºC, but much less viscous at 32 ºC (Figure 4.4A), since it did not undergo sol-gel transition at all in the studied temperature range (20 to 42 ºC, Figure 4.4B). 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 TG_{P+FOS} (the respective thermosensitive gel, with a composition equal to its external phase) (Figure 4.4B), which indicated that emulsification delayed the gelation process, in relation to the thermosensitive gel alone. Likewise, the T_aMax of the nanoemulgel was higher than the respective thermogel (Table 4.3), 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 4.3) 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 4.3, as well as the remaining characterization parameters for TG_{P+FOS} and TN_{P+FOS} 4:6.

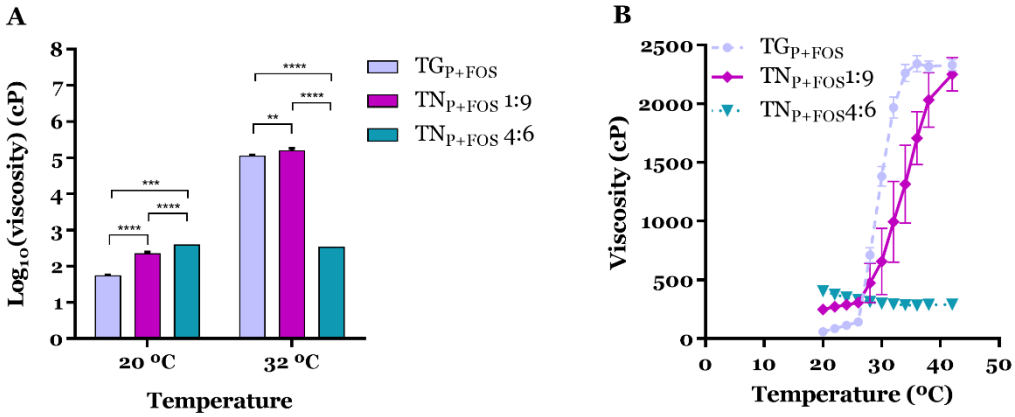

Figure 4.4. Rheological characterization of the nanoemulgels and the respective Pluronic thermosensitive gel. Logarithm of zero shear viscosity at different temperatures (A); temperature effect at constant shear rate (80 s^{-1}) (B); data are shown as mean ± SEM of 3 independent formulation batches for TN_{P+FOS} 1:9 and TN_{P+FOS} 4:6, and of a representative batch for TG_{P+FOS}; FOS – fosphenytoin; P – Pluronic; SEM – standard error of the mean; TG – thermosensitive gel; TN – thermosensitive nanoemulgel. *** $p < 0.001$, **** $p < 0.0001$ in two-way ANOVA analysis and a Tukey's multiple comparisons post-test, applied after a Log_{10} transformation of the viscosity values.

Table 4.3. Osmolality, mean size, PDI, 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).

		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**	599.6	n.d.	n.d.	n.d.	57.43	116145	29.5	27.6
	SD	14.5				1.64	- 1810; + 1838	0.1	
	n	5				3	3	3	
	RMPS	3				1	1	1	
	R^2	-				-	0.9995	0.9985	
TN_{P+FOS} 1:9	**Mean**	1375.0	219.7	0.237	-10.7	233.31	168655	33.7	30.3
	SD	52.4	26.8	0.040	2.7	0.42	- 11492; + 12333	0.2	
	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.	407.52	352.02	#	#
	SD		6.0	0.018		0.89	2.33		
	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 – linear regression's coefficient of determination; RMPS - replicate measurements per sample, contemplated 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/no gelling occurred.

4.3.3. Drug release study

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 assessed the drug release from an aqueous drug solution having fosphenytoin at 1.3 mg/g, phenytoin at 0.2 mg/g and Transcutol at 30% (FPT solution). Percentual drug release and percentual drug release rates are shown in Figure 4.5 and Table 4.4. 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%) and also in N_{FOS} 4:6 (13.6%), since N_{FOS} 1:9 had less cosolvent (only 3.4%). 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.

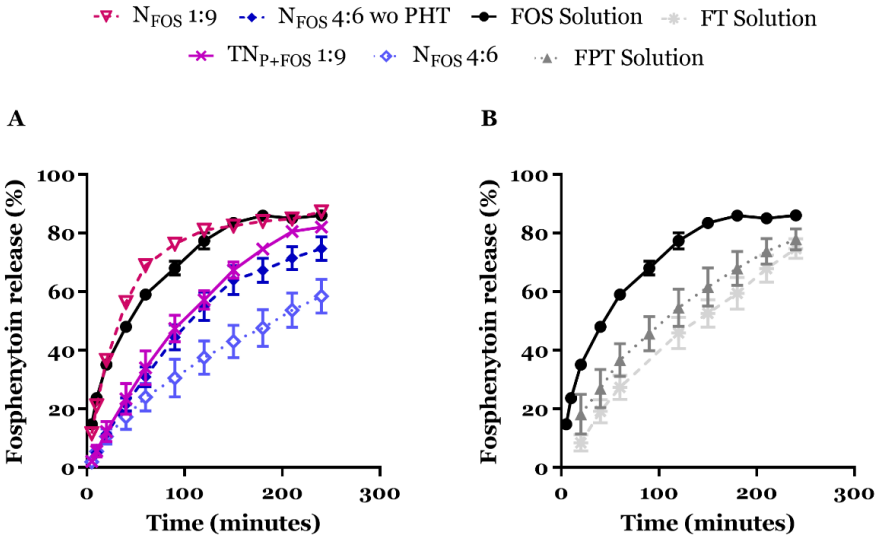

Figure 4.5. Fosphenytoin's percentual drug release between 5 and 240 minutes, for nanoformulations (A) and solutions (B). The aqueous solution containing fosphenytoin only (FOS solution) appears in both graphics, since it is the most reliable positive control. FOS – fosphenytoin; FPT – fosphenytoin, phenytoin and Transcutol aqueous solution; FT – fosphenytoin and Transcutol aqueous solution; N – nanoemulsion; P – Pluronic; PHT – phenytoin; TN – thermosensitive nanoemulgel; wo - without.

Table 4.4. 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 = √X) versus percentual drug release divided by the area of the membrane used in the assay (Y = Y/0.64). Slopes then compared using an F test.

Drug	Formulation	R^2 ($R^{2'}$)	Drug release rate (%·cm^{-2}·min$^{-1/2}$)		Significance of differences in rate between formulations (p value)					
			Mean	SD	N_{FOS} 1:9	N_{FOS} 4:6	N_{FOS} 4:6 wo PHT	FPT Solution	FOS Solution	FT Solution
Fosphenytoin	**TN_{P+FOS} 1:9**	0.9964 (0.9809)	10.4	0.3	****	****	NS	NS	0.0122	NS
	N_{FOS} 1:9	0.9980 (0.9901)	16.5	0.5		****	****	****	****	****
	N_{FOS} 4:6	0.9982 (0.8791)	6.6	0.4			****	0.0210	****	0.0006
	N_{FOS} 4:6 wo PHT	0.9924 (0.9477)	9.8	0.5				NS	0.0157	NS
	FPT Solution	0.9992 (0.8258)	8.8	0.9					0.0306	NS
	FOS Solution	0.9969 (0.9909)	12.3	0.3						0.0104
	FT Solution	0.9982 (0.9270)	9.2	0.6						
Phenytoin	**TN_{P+FOS} 1:9**	0.9770 (0.8173)	5.7	0.7	0.0301	NS		NS		
	N_{FOS} 1:9	0.9981 (0.9690)	7.1	0.2		****		NS		
	N_{FOS} 4:6	0.9972 (0.6578)	4.0	0.6				0.0032		
	FPT Solution	0.9900 (0.6909)	8.1	1.3						

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

A fosphenytoin aqueous solution (FOS solution, with no phenytoin and no Transcutol) and a fosphenytoin plus Transcutol aqueous solution (FT solution, with no phenytoin) were also tested to better clarify the previous results (Figure 4.5B). 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 wo PHT) (Figure 4.5A). 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 wo 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 4.6 and Table 4.4), most results were as expected, since FPT solution was the fastest, followed by N_{FOS} 1:9, and with TN_{P+FOS} 1:9 and N_{FOS} 4:6 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.

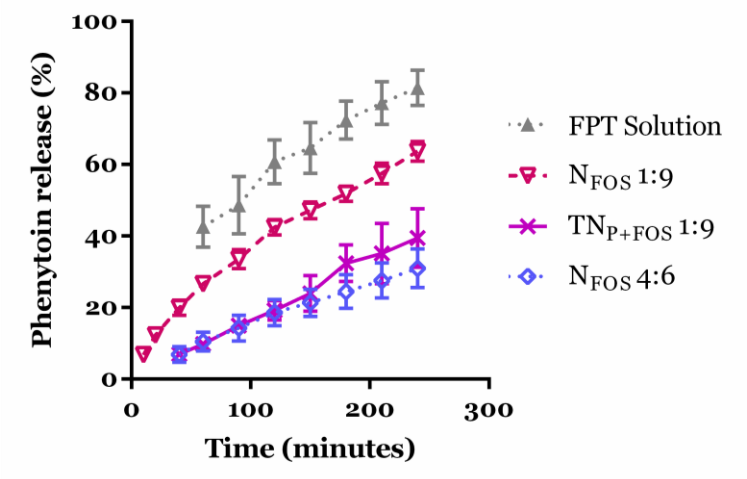

Figure 4.6. Phenytoin percentual release between 5 and 240 minutes. FOS – fosphenytoin; FPT – fosphenytoin, phenytoin and Transcutol aqueous solution; N – nanoemulsion; P – Pluronic; TN – thermosensitive nanoemulgel.

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 through the membrane, or be transported inside the droplets themselves, and although droplet mean size may be around 200 nm, a considerable amount of droplets might not pass (due to being bigger, because of heteretogeneity).

In what concerns cumulative drug quantity (Figure 4.7), 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 the best choice. 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.

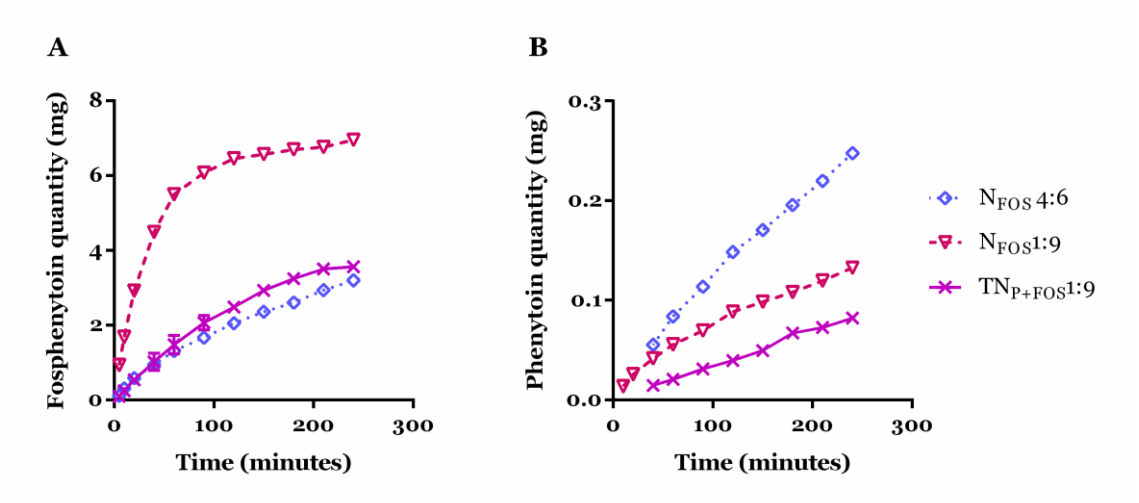

Figure 4.7. Cumulative drug quantity release for the most relevant formulations, for fosphenytoin (A) and phenytoin (B). FOS – fosphenytoin; N – nanoemulsion; P – Pluronic; TN – thermosensitive nanoemulgel.

As for the cumulative drug quantity release rate (Table 4.5), 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.

Table 4.5. 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 = √X) versus cumulative drug quantity divided by the area of the membrane used in the assay (Y = Y/0.64). Slopes were compared using an F test.

Drug	Formulation	R^2 (R^2')	Drug release rate ($mg \cdot cm^{-2} \cdot min^{-1/2}$)		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**	0.9964 (0.9809)	0.454	0.015	****	0.0099
	N_{FOS} 1:9	0.9980 (0.9901)	1.315	0.037		****
	N_{FOS} 4:6	0.9982 (0.8791)	0.362	0.024		
Phenytoin	**TN_{P+FOS} 1:9**	0.9758 (0.8135)	0.012	0.002	0.0260	0.0024
	N_{FOS} 1:9	0.9981 (0.9693)	0.015	0.001		****
	N_{FOS} 4:6	0.9970 (0.6575)	0.032	0.005		

FOS – fosphenytoin; FPT – fosphenytoin, phenytoin and Transcutol aqueous solution; FT – fosphenytoin and Transcutol aqueous solution; N – nanoemulsion; P – Pluronic; PHT – phenytoin; R^2 – linear regression's coefficient of determination, using mean values for each time point; R^2' – linear regression's coefficient of determination, using all individual values corresponding to each individual 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. Nevertheless, the achieved drug strength was, for all final formulations, still lower than that of the fosphenytoin formulations (34.8 mg/g of phenytoin equivalents, Chapter 3).

4.4. 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 seems to be important in order to have phenytoin in the soluble form, even in emulsions, although apparently 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 reduce 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, however, led to a steep increase in osmolality, since it is a disodium salt, limiting its concentration for potential safety reasons.

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 could be addressed in future studies. However, since final drug strength was still lower than the obtained for the fosphenytoin formulations, there still seemed to be scope for improvement, and thus a second phase of nanometric emulsion development was attempted, as described in the following Chapter 5.

Chapter 5 – Microemulsions of phenytoin and fosphenytoin

5.1. Chapter overview and main objectives

In Chapter 3, the use of phosphate ester prodrugs like fosphenytoin as an efficient and safe strategy to increase the intranasal bioavailability of poorly soluble drugs was demonstrated. The developed formulations reached a drug strength of 34.8 mg/g of phenytoin equivalents, and the formulation containing HPMC and albumin prolonged drug concentration in the brain over time and led to an increased absolute bioavailability. Moreover, since it was not possible to develop formulations containing phenytoin alone (not without the use of great amounts of organic solvents), in Chapter 4 we formulated both fosphenytoin and phenytoin together in oil-in-water nanoemulsions and nanoemulgels, aiming to develop preparations that could result in a faster and/or more effective brain targeting, by having a fraction of phenytoin in the active and diffusible form. Nevertheless, these formulations' drug strength only reached between 22 and 27 mg/g of phenytoin equivalents, a value lower than the obtained for the simpler fosphenytoin formulations.

Hence, the work shown in this chapter aimed to develop nano or microemulsions, containing the combination of phenytoin and fosphenytoin, with increased total drug strength, while having the maximum amount of drug in the active form. The formulations were characterized in what concerned viscosity, osmolality, pH, droplet mean hydrodynamic size, polydispersity index, zeta potential, and *in vitro* drug release. The selected formulations' *in vivo* pharmacokinetic profile was then determined and compared to the previously obtained results for the simpler fosphenytoin formulations.

5.2. Materials and methods

5.2.1. Materials

Fosphenytoin disodium (USP) was purchased from Jai Radhe Sales (Ahmedabad, India), provided as a hydrated disodium salt, however throughout the text mass concentration will be indicated as calculated for the anhydrous acid form. Fosphenytoin and phenytoin (USP) reference standards, ketoprofen, monobasic sodium phosphate, bovine serum

albumin and Kolliphor® EL (polyoxyl 35 castor oil) were bought from Sigma-Aldrich (Steinheim, Germany). High-performance liquid chromatography (HPLC) grade methanol, analytical grade triethylamine and diethyl ether, and sodium chloride and sodium hydrogen carbonate were acquired from Fisher Scientific (Leicestershire, United Kingdom). Sodium acetate was bought from Merck (Darmstadt, Germany), potassium chloride from Chem-Lab (Zedelgem, Belgium), and dibasic sodium phosphate from Acros Organics (Geel, Belgium). Pentobarbital sodium injection solution (Eutasil®) was acquired from Ceva (Libourne, France) and magnesium chloride and sodium hydroxide were purchased from Labkem (Barcelona, Spain). Calcium chloride and ortho-phosphoric acid 85% (v/v) were acquired from Panreac (Barcelona, Spain), and hydrochloric acid 37% (v/v) and polyethylene glycol 300 were bought from Fluka (Seelze, Germany). Castor oil, Cetiol® V (decyl oleate), glycerol, Miglyol® 812 (medium chain triglycerides), soybean oil, polyethylene glycol 400, propylene glycol, Span® 80 (sorbitan monooleate), Tween® 20 (polysorbate 20), Tween® 60 (polysorbate 60) and Tween® 80 (polysorbate 80) were purchased from Acofarma (Barcelona, Spain). Capryol® 90 (propylene glycol monocaprylate, type II), Labrafac™ PG (propylene glycol dicaprylocaprate), Labrasol® ALF (caprylocaproyl polyoxyl-8 glycerides) and Transcutol® HP (highly purified diethylene glycol monoethyl ether) were gift samples from Gatefossé (Lyon, France). Dynacet® 285 (diacetylated monoglycerides), Imwitor® 948 (glycerol mono-oleate) and Imwitor® 988 (glycerol monocaprylate) were gift samples from IOI Oleochemical (Hamburg, Germany), and Kolliphor® RH40 (polyoxyl 40 hydrogenated castor oil) was a gift sample from BASF (Ludwigshafen, Germany). Water was of ultra-pure grade (Milli-Q water apparatus, 0.22 μm filter, Merck, Darmstadt, Germany).

5.2.2. Emulsions preparation and characterization

Phenytoin was dissolved in the emulsions' preconcentrates before emulsification, which were prepared by weighing together the oil, surfactant, cosurfactant and/or cosolvent. The aqueous phase was made of either water (vehicle studies) or a fosphenytoin plus albumin aqueous solution (drug formulations), and its pH was adjusted to 6 - 7 (nasal pH) (Orion Star A211 pH meter, Thermo Fisher Scientific, Massachusetts, United States of America). Then the emulsions were prepared by weighing the preconcentrate and adding a small part of the aqueous phase first (to create a water-in-oil emulsion), followed by mechanical or magnetic stirring, and then adding the rest of the aqueous phase (to invert the emulsion to the desired oil-in-water system), followed by more

stirring. Final formulations' pH was verified using universal indicator paper (Nahita, Auxilab S.L., Navarra, Spain). All formulation components were weighted together, and final concentrations in the text are percentual w/w concentrations [% (w/w)], indicated as percentage only (%) for simplification purposes, unless indicated otherwise.

The composition of the formulations selected from initial vehicle studies, chosen for drug incorporation, is shown in Table 5.1.

Table 5.1. Composition of emulsions selected for phenytoin incorporation at 5 mg/g. Final formulations' concentrations are indicated as percentual w/w values.

Component	**Excipient**		**Formulation code name**					
	Class	**Name**	**F1**	**F2**	**F3**	**F4**	**F5**	**F3-FOS**
Pre-concentrate	**Oil**	**Capryol 90**	8.66 %	17.33 %	24.75 %	29.70 %	-	25.00 %
		Imwitor 988	-	-	9.90 %	-	-	10.00 %
		Miglyol 812	-	-	-	-	9.90 %	-
	Hydrophilic Surfactant	**Tween 20**	25.99 %	-	-	2.48 %	-	-
		Kolliphor EL	-	17.32 %	14.85 %	17.32 %	-	15.00 %
		Tween 80	-	-	-	-	22.77 %	-
	Cosolvent	**Transcutol HP**	14.85 %	14.85 %	-	-	16.83 %	-
	Drug	**Phenytoin**	0.50% (5 mg/g)					-
Aqueous phase	**Drug**	**Fosphenytoin**	4.25% (42.5 mg/g)					
	Polymer	**Albumin**	2.00%					
	Purified water		Qs 100%					

F4 [149] and F5 (chapter 4) were adapted from previously developed formulas.

Viscosity measurements were done using a cone-plate rheometer (DV3T, Brookfield Ametek, Massachusetts, USA), with a CP40Z spindle and a sample volume of 0.5 mL. Measurement temperature was regulated through a thermostated water bath (MultiTemp III Thermostatic Circulator, Thermo Fisher Scientific, New Hampshire, USA), and was set to a constant value – either 20 ºC (mean room temperature) or 32 ºC (mean nasal cavity temperature). After confirmation of the Newtonian behavior of the formulations, viscosity was measured at the highest rotational speed (within the apparatus measurement range), for lower associated measurement error, and each batch was measured only once.

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

Emulsion's droplet mean hydrodynamic size (simply referred to as "mean size" throughout the text) and PDI, obtained by cumulants' analysis of dynamic light scattering data, and zeta potential, determined by electrophoretic light scattering, were measured using a Zetasizer Nano ZS apparatus (Malvern, United Kingdom). Samples were diluted 50-fold in water (vehicle studies) or phosphate buffer (20 mM, at pH 7, for drug formulations), and measured at 25 ºC in disposable ultra-violet/visible polymethyl methacrylate cuvettes (Kartell, Noviglio, Italy). Zeta potential was measured using a Dip Cell (ZEN 1002, Malvern, United Kingdom). Analysis was performed automatically three times for each sample, in at least two different samples of each batch.

5.2.3. Phenytoin solubility tests

In order to achieve maximum phenytoin solubility in the developed vehicles, a preliminary solubility test was done in a series of individual excipients. An excess of phenytoin powder was added to 1 g of each excipient. These mixtures underwent mechanical stirring for 24 h at room temperature. Afterwards, the tubes were centrifuged at 13500 rpm for 5 minutes, and a sample was taken from the supernatant and diluted 5000-fold in three steps, carefully considered to not let the solubilized phenytoin precipitate and to avoid phase separation: the first dilution was done in pure methanol or Transcutol (20-fold), the second in Transcutol at 30% (50-fold), and the third in water (5-fold). Between dilutions the tubes were vortexed and centrifuged under the same conditions as before. Samples were then analyzed by HPLC, using a previously developed and validated method (Chapter 4, section 4.2.5). Excipients alone, with no solubilized drug, were also processed in the same way, to assess for possible interference in the quantification.

After defining final emulsion composition, phenytoin solubility was also assessed in selected formulations, using the same steps as for the individual excipients. Vehicle and albumin interference in the quantification method was also determined.

5.2.3.1. High-performance liquid chromatography conditions

Chromatographic apparatus comprised a HPLC system (LC-2010A HT Liquid Chromatography) coupled with a diode-array detector (SPD-M20A), with automatic control of the instrumental parts by the data acquisition software (LabSolutions, version 5.52) (Shimadzu, Kyoto, Japan). Analyte separation was achieved by means of a reversed-phase guard column (C18, 5 μm particle size, 4 × 4 mm) attached to a reversed-phase column (C18, 3 μm particle size, 55 × 4 mm) (LiChroCART® Purospher® STAR models, Merck, Darmstadt, Germany), and performed at 30 ºC. The 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, with elution being done in isocratic mode at 1 mL/min. The injection volume was 20 μL, analyte separation was achieved within 20 minutes of each run and detection was done at 215 nm.

5.2.4. *In vitro* drug release assay

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

Experimental protocol was based on a previously developed method (Chapter 4, section 4.2.5), with slight modifications. The bottom chamber was filled with 1.8 mL of nasal fluid simulant buffer, plus albumin at 2%, having a pH of 6.5. Once the chambers were fully assembled, 50 μL of this buffer solution were placed on the upper side of the membrane, until the intended temperature was reached. After that, the buffer on the membrane was removed and replaced with 50 μL of the formulation. The bottom chamber fluid was homogenized through magnetic stirring (Micro Stirring Bars, 2 mm, VWR, United Kingdom), and samples of 100 μL were taken from it at 5, 10, 20, 40, 60, 90, 120, 150, 180, 210 and 240 minutes, with the volume being replaced with new buffer solution at each time. Drug release from positive controls was also assessed, one for each drug, at concentrations similar to those found in the developed formulations: phenytoin at 5 mg/g, dissolved in Transcutol, and fosphenytoin at 42.5 mg/g, dissolved in water. Following sample collection there was a two-step sample dilution: the first dilution (20-fold) was done in Transcutol at 30%, and the second dilution (5-fold) was done in buffer solution. For the quantification of initial drug concentration in the formulations the first dilution was higher (400-fold). Sample quantification was then done by HPLC, using a previously developed and validated method, as described in section 5.2.3.

5.2.5. *In vivo* pharmacokinetic study

5.2.5.1. Animal experimentation

Animal experimentation studies included adult male CD-1 mice from our own institution's certified animal facility, housed under controlled environmental conditions (12 h light/dark cycle, 20 ± 2 ºC, 50 ± 5% relative humidity) and with free access to tap water and standard rodent diet (4RF21, Mucedola, Italy). Their age ranged between 8 and 9 weeks and their weight between 25 and 43 g. All animal procedures were done in conformity with the regulations from the European Directive 2010/63/EU, regarding the protection of laboratory animals used for scientific purposes, and approved by the Local Animal Ethics Committee and by the competent national authority [Portuguese National Authority for Animal Health, Phytosanitation and Food Safety (DGAV – Direção Geral de Alimentação e Veterinária)].

Before drug administration each mouse was anesthetized through intraperitoneal injection (60 mg/kg of pentobarbital). For the administrations, which were all intranasal, the mouse's body was placed on top of a heating pad, on its left side, and a flexible catheter, attached to a 50 µL syringe (Hamilton, Nevada, USA), was inserted 3 to 4 mm into the right nostril. After drug administration the mice were left to recover from anesthesia in a temperature-controlled environment, in a supine position.

There were two experimental groups, of 44 animals each (11 time points – 5, 15, 30, 60, 120, 240, 360, 480, 720, 960 and 1440 minutes, 4 mice per time point). In one group a microemulsion containing phenytoin and fosphenytoin (F3) was administered intranasally, and in the other group a microemulsion containing fosphenytoin only (F3-FOS) was also administered intranasally. The administered dose was of 5.8 mg/kg of phenytoin equivalents for both groups (maximum of 5.9 µL per 30 g of mouse body weight).

5.2.5.2. Sample collection, processing and analysis

After euthanasia mice blood and brain were collected, processed and analyzed using a previously developed and validated method (Chapter 3, section 3.2.5.2). Subsequently to being collected and manually stirred inside tubes containing ethylenediaminetetraacetic acid (1 mL capacity, with K_3 EDTA, FL Medical, Italy), 300 µL of blood were transferred and mixed with 300 µL of orthophosphoric acid 85% (v/v) inside an eppendorf tube, making a 1:1 blood-to-acid mixture ratio (v/v). The addition of orthophosphoric acid was

meant to prevent fosphenytoin conversion to phenytoin (enzyme deactivation). With that same purpose, whole brains were homogenized (Ika Ultra-Turrax® T25 Basic, Staufen, Germany) in a mixture of water and orthophosphoric acid, also in a 1:1 (v/v) ratio (1 g of tissue per 4 mL of mixture). Brain homogenates were then centrifuged at 14000 rpm, 4 ºC, for 10 minutes (MIKRO 200R microcentrifuge, Hettich, Tuttlingen, Germany), and the supernatant was kept. All samples were put on ice until stored. Acidified blood and acidified brain homogenates' supernatants were stored at -20 ºC (RZ80FHRS freezer, Samsung, Seoul, South Korea) until needed.

All samples were kept on ice during processing as well, and the experimental protocol consisted of adding 20 µL of ketoprofen (the internal standard) spiking solution to either 100 µL of brain homogenate supernatant sample or 200 µL of blood sample. Then followed liquid-liquid extraction with diethyl ether (addition of 1000 µL to each sample), vortexing for 30 seconds and centrifugation for 5 minutes, at 13500 rpm, at room temperature (microcentrifuge, Gyrozen, Daejeon, South Korea), with the resulting organic phase being transferred to a glass tube, and the aqueous phase being re-extracted twice more, under the same conditions. The combined organic phases were then evaporated to dryness under a gas stream at 45 °C, and reconstituted with 100 µL of mobile phase.

The chromatographic apparatus and analyte separation conditions were the same as for the drug solubility and drug release studies (section 5.2.3.1), but instead of having a phosphate buffer the mobile phase was made of 64% sodium acetate buffer (10 mM, pH 5, with 0.25% triethylamine) plus 36% methanol. Fosphenytoin and phenytoin detection was still done at 215 nm, but the detection of the internal standard was done at 280 nm.

5.2.6. Data analysis

Formulation and drug release data analysis was done using Prism software, version 8.0, from GraphPad, with the significance level set at 0.05.

A two-way ANOVA, followed by a Tukey's multiple comparisons post-test was used to compare mean size and PDI values over time after preparation (short-term stability). A one-way ANOVA, followed by a Tukey's multiple comparisons post-test was used to compare mean viscosity values between different formulations, and between different temperatures for the same formulation. A *t*-test was used to compare mean size and PDI mean values between different formulations.

Drug release parameters were determined taking into account initial drug strength. Drug release rate was calculated through the Higuchi model [133,134], in which both time (X) and drug release percentage (Y) were transformed: X by calculating its square root ($X = \sqrt{X}$) and Y by dividing it by the area of the membrane used in the assay ($Y = Y/0.64$). A linear regression was applied after this transformation, and the drug release rate corresponded to the obtained slopes. Late time points with values out of the linear zone were excluded, and the slopes were compared two-by-two using an F-test to assess for significant differences between formulations.

Differences between formulations' drug concentration levels in blood and brain were determined by two-way ANOVA analysis, with Tukey's multiple comparisons post-test. Time points that did not coincide between the two pharmacokinetic studies (the one presented in this chapter and the one shown in Chapter 3) were excluded from this analysis.

The calculation of pharmacokinetic parameters was done using the add-in program for Microsoft Excel "PKSolver". The analysis was non-compartmental, with the administrations being considered extravascular. Blood and brain maximum drug concentration (C_{max}) and time to reach it (T_{max}) were directly derived from the experimental data. The area under the drug concentration vs time curve from time zero to the last quantifiable drug concentration ($AUC_{0\text{-}t}$) was determined by the linear trapezoidal method. The area under the drug concentration vs time curve from time zero to infinity ($AUC_{0\text{-}inf}$) was calculated by adding $AUC_{0\text{-}t}$ to the last quantifiable drug concentration (with adequate precision and accuracy, C_{last}) divided by the elimination rate constant (k_{el}) (the formula being $AUC_{0\text{-}inf} = AUC_{0\text{-}t} + C_{last}/k_{el}$). k_{el} was estimated by applying a log-linear regression to the terminal segment of the drug concentration vs time curve. The elimination half-life ($t_{1/2el}$) was calculated by dividing ln2 by k_{el} (the formula being $t_{1/2el} = \ln 2/k_{el}$), and the mean residence time (MRT) by dividing the area under the first moment curve (AUMC) by the $AUC_{0\text{-}inf}$ (the formula being MRT = AUMC/ $AUC_{0\text{-}inf}$). The percentage of the AUC that was extrapolated from the last quantifiable drug concentration to infinity [AUC_{extrap} (%)] was also calculated.

Absolute blood bioavailability ($B\%_{blood\ IN/IV}$) of the selected intranasally administered emulsions was calculated using equation 5.1:

$$B\%_{blood\ IN/IV} = \frac{AUC_{\ blood_{IN}}}{AUC_{\ blood_{IV}}} \times 100 \quad (5.1).$$

$AUC_{blood\ IV}$ values correspond to an intravenous fosphenytoin solution (previous study, Chapter 3).

Relative brain and blood bioavailability ($RB\%_{brain}$ and $RB\%_{blood}$, respectively) were also calculated for these emulsions, with equations 5.2 and 5.3:

$$RB\%_{brain} = \frac{(AUC\ _{brain_{IN}})_{emulsion}}{(AUC\ _{brain_{IN}})_{solution}} \times 100 \qquad (5.2)$$

$$RB\%_{blood} = \frac{(AUC\ _{blood_{IN}})_{emulsion}}{(AUC\ _{blood_{IN}})_{solution}} \times 100 \qquad (5.3)$$

$(AUC_{brain\ IN})_{solution}$ and $(AUC_{blood\ IN})_{solution}$ values correspond to a simple intranasal aqueous fosphenytoin solution (previous study, Chapter 3).

Moreover, in order to compare the results obtained with the developed emulsions to the intranasal formulation with which we obtained the best results in the previous study (fosphenytoin formulation containing HPMC and albumin, Chapter 3), we calculated relative brain and blood bioavailability considering AUC values from that same formulation ($RB\%_{brain\ best}$ and $RB\%_{blood\ best}$, respectively), using formulas 5.4 and 5.5:

$$RB\%_{brain\ best} = \frac{(AUC\ _{brain_{IN}})_{emulsion}}{(AUC\ _{brain_{IN}})_{best\ formulation}} \times 100 \qquad (5.4)$$

$$RB\%_{blood\ best} = \frac{(AUC\ _{blood_{IN}})_{emulsion}}{(AUC\ _{blood_{IN}})_{best\ formulation}} \times 100 \qquad (5.5)$$

5.3. Results and discussion

5.3.1. Formulation development with assessment of drug solubility, rheology, osmolality and droplet size characteristics

In order to maximize the amount of phenytoin in the emulsions (in the active form, not as prodrug) we did a preliminary solubility screening in individual excipients. The highest phenytoin solubility was obtained with Capryol 90 and Imwitor 988 in the hydrophobic excipients category, with Tween 20, Tween 80 and Kolliphor EL in the hydrophilic surfactants category, and with Transcutol HP in the cosolvent category (Figure 5.1). Therefore, these excipients were selected and mixed in different proportions in order to produce nano or microemulsions with good characteristics (homogeneous, with small mean size and visually stable for at least a few hours). To maximize the

amount of phenytoin in the final formulations, the amount of oil phase was maximized, and therefore the preconcentrate-to-aqueous phase proportion was maintained at 50:50.

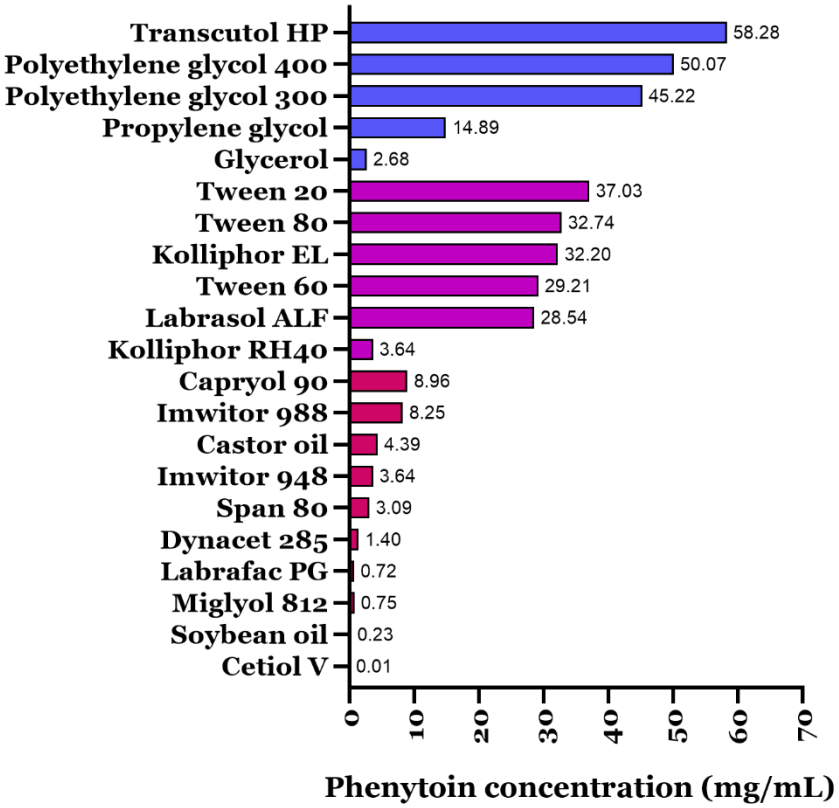

Figure 5.1. Phenytoin solubility screening test in individual excipients. One batch for each excipient, one measurement only. Blue bars correspond to cosolvents, purple bars to hydrophilic surfactants, and maroon bars to hydrophobic excipients.

A few series of vehicle formulas (without drugs) were screened regarding droplets mean size and PDI, in order to select a small number with different composition and acceptable droplet size (data not shown). In the selected vehicles there was a general tendency for mean size decreasing with increasing dilution before size measurement, which is a known characteristic of microemulsions. Moreover, the chosen formulas had mean sizes within the characteristic microemulsion's range (10-100 nm, data not shown) [150].

In the vehicles containing Capryol 90 (here used as the oil), Tween 20 (hydrophilic surfactant) and Transcutol (cosolvent), decreasing the oil-to-surfactant ratio to less than 1 made the emulsions more homogeneous (decreased PDI) and the mean droplet size smaller. In contrast, in the vehicles containing Capryol 90 (oil), Kolliphor EL

(hydrophilic surfactant) and Transcutol (cosolvent), increasing the oil-to-surfactant proportion was what decreased the PDI and mean size, and the best ratios were ≥ 1.

Having the decrease of the potential toxicity of the formulations in mind, we also aimed for formulas that did not have any cosolvent. Therefore, we developed vehicles containing Capryol 90 (oil), Tween 20 or Kolliphor EL (hydrophilic surfactant) and Imwitor 988 (hydrophobic surfactant). Results were better for Kolliphor EL than for Tween 20, and a hydrophobic excipients-to-hydrophilic surfactant ratio > 1 seemed to be beneficial. Here, a high Capryol 90 quantity was needed for good homogeneity.

In all the developed formulas, replacing Tween 20 or Kolliphor EL with Tween 80 increased the PDI. Therefore, this excipient was excluded from further studies.

Additionally, in the existing scientific literature we also discovered an already developed formula using some of the selected excipients: Capryol 90 (oil), Tween 20 and Kolliphor EL (hydrophilic surfactants) [149]. Since it had a droplet size within the microemulsion range (around 40 nm) and a reasonable PDI value (0.3 to 0.4), we sought to adapt it using our experimental conditions.

Since all the developed (or adapted) vehicle formulas were, apparently, microemulsions, we also adapted a formula of a nanoemulsion that we previously developed, containing Miglyol 812 (oil), Tween 80 (hydrophilic surfactant) and Transcutol (cosolvent) (Chapter 4). This formula required extra homogenization through premix membrane emulsification, but was otherwise stable (for at least 2 weeks), relatively homogeneous (PDI < 0.3) and had a small mean size (≈ 200 nm), within the nanoemulsion range (20-200 nm) [150]. Nevertheless, for this study we changed the preconcentrate-to-water phase proportion, since in the previous study it was either 10:90 or 40:60, and here we set it 50:50 (again, to maximize phenytoin content).

After choosing a set of preconcentrates with different composition, a second solubility study was then done to assess how much phenytoin could be solubilized in the resulting microemulsions, with fosphenytoin in the external phase (at 43.1 mg/g, isotonic concentration) plus albumin at 2% (concentration in the final formulation). For the previously developed nanoemulsion, F5, phenytoin concentration had already been established at 10 mg/g in the preconcentrate, originating 5 mg/g in the nanoemulsion as prepared in present work. Even though it had Tween 80 and Transcutol in its composition (some of the best excipients in solubilizing phenytoin), the oil was Miglyol 812, that had a phenytoin solubility over 10 times lower than that of Capryol 90. Hence,

the developed microemulsions were expected to solubilize moderately more than 5 mg/g. Nevertheless, results showed that, despite efforts to choose the excipients that could individually solubilize phenytoin the most, its solubility was for all microemulsions between 5 and 6 mg/g (Figure 5.2), which is almost the same amount that F5 was able to solubilize. Since that in the solubility test we reach saturation, we decided on having the slightly lower concentration of phenytoin, setting it at 5 mg/g for all the developed microemulsions (10 mg/g in the preconcentrate), same as for the F5 nanoemulsion.

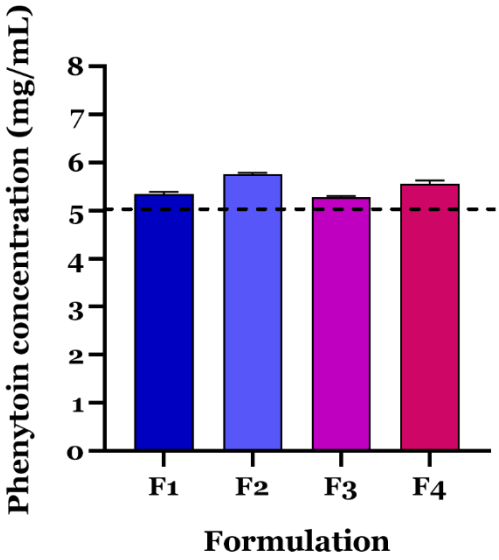

Figure 5.2. Phenytoin solubility test in the microemulsions. One batch for each different formulation, 5 measurements per formulation. Data are presented as mean ± SEM. SEM – standard error of the mean.

Since it was not possible to increase the amount of phenytoin solubilized in the microemulsions as compared with our previous work, in order to reach a total drug strength of at least 34.8 mg/g of phenytoin equivalents (same as for the previous study, with the formulations containing fosphenytoin only), we tried increasing the fosphenytoin amount (solubilized in the external phase) to 42.5 mg/g (concentration in the final formulation, 85 mg/g in the external phase). The external phase was no longer isotonic, having an osmolality of around 500 mOsmol/kg, but it was still within the established limits for marketed nasal preparations [136]. With phenytoin at 5 mg/g and fosphenytoin at 42.5 mg/g, final drug strength was 34.6 mg/g of phenytoin equivalents, which is very near the strength of the formulations used in the previous *in vivo* study. Therefore, we assessed the suitability of adding the drug and prodrug at these concentrations, plus albumin at 2% (dissolved at 4% in the external phase), to formulations F1 to F5 (all prepared with a preconcentrate-to-external phase proportion of 50:50). However, formulations F1 and F5 were not compatible with drug

incorporation (plus albumin) into the vehicle, at least at the selected concentrations, since they showed immediate physical instability upon addition of the external phase (containing fosphenytoin and albumin) to the preconcentrate (containing phenytoin), forming a precipitate and showing phase separation. These formulations were, therefore, excluded. Microemulsions F2, F3 and F4 were visually homogeneous. However, F4 had PDI values between 0.5 and 0.6, thus having a highly heterogeneous size, and therefore it was excluded as well.

On the contrary, formulations F2 and F3 had good PDI values, with relatively small mean sizes (Table 5.2). Both formulations were considered to be Newtonian at 20 and 32 ºC, and had a higher viscosity at room temperature than at nasal cavity temperature ($p < 0.0001$). F2 had lower mean size ($p < 0.01$), PDI (no statistical significance) and viscosity ($p < 0.0001$) values than F3 (Table 5.2). Zeta potentials were basically considered neutral (data not shown), as expected due to the excipients also being neutral.

Table 5.2. Mean size, PDI and viscosity (at 20 and 32 ºC) characterization of the formulations selected for drug release studies. Three batches of each different formulation were used. One or two measurements per batch were performed (for viscosity, or mean size and PDI, respectively). Data are presented as mean ± SEM.

Formulation name	Mean size (nm)	PDI	Viscosity at 20 ºC (cP)	Viscosity at 32 ºC (cP)
F2	60.9 ± 2.2	0.150 ± 0.015	75.97 ± 0.71	43.33 ± 0.32
F3	83.2 ± 3.7	0.198 ± 0.023	96.58 ± 1.45	58.44 ± 1.09

PDI – polydispersity index; SEM – standard error of the mean.

As for short-term physical stability, F3 was stable for at least 1 week (Figure 5.3), but not 2 weeks, since there was a significant increase in mean size and PDI values at the end of the second week ($p < 0.0001$). In what concerns mean size and PDI measurements, F2 seemed stable for at least 2 weeks, but a small precipitate formed about 48 h after preparation, and thus this was considered to be its stability time span. Nevertheless, for the purpose of doing further studies (*in vitro* drug release and *in vivo* pharmacokinetics) 1 week and 48 h stabilities were enough to guarantee the good condition of the formulations until used.

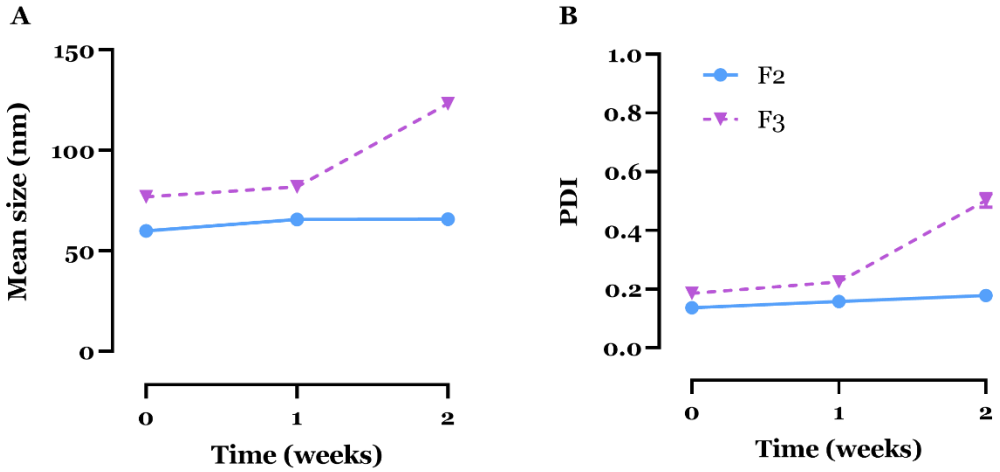

Figure 5.3. Mean size and PDI values' variation from 0 to 2 weeks. One batch for each different formulation, three measurements per time point. PDI – polydispersity index; SEM – standard error of the mean.

It is important to point out that, when compared to the previously developed nanoemulsions (Chapter 4), these microemulsions were easier to prepare, since they did not need additional homogenization through premix membrane emulsification, having spontaneous formation upon mixing the external phase with the preconcentrate, which is an advantage. As for osmolality, it ranged between 2000 and 2400 mOsmol/kg, which is quite high. This might be due to the fact that, since these formulations are microemulsions, there is a great number of very small droplets or dissolved components contributing to these values. Nevertheless, considering the present objective (hypothesis testing) it was important to maximize phenytoin's strength, so we proceeded with these formulations (F2 and F3) for further studies. Despite both being microemulsions, and having reasonably similar mean size and viscosity values, F2 had Transcutol and F3 did not. In our previous study with nanoemulsions the *in vitro* drug release results suggested that Transcutol could slow down and/or inhibit fosphenytoin and phenytoin drug release (Chapter 4). We sought to confirm if the same happened for these microemulsions in the following section 5.3.2.

5.3.2. *In vitro* drug release

The *in vitro* drug release profiles of the selected formulations – F2 and F3 – were evaluated using horizontal Ussing chambers. Two positive controls were also evaluated for drug release, one for each drug – a phenytoin solution in pure Transcutol (drug at 5 mg/g) and a fosphenytoin solution in water (drug at 42.5 mg/g). The drug release

percentage profiles and calculated drug release rates are shown in Figure 5.4 and Table 5.3.

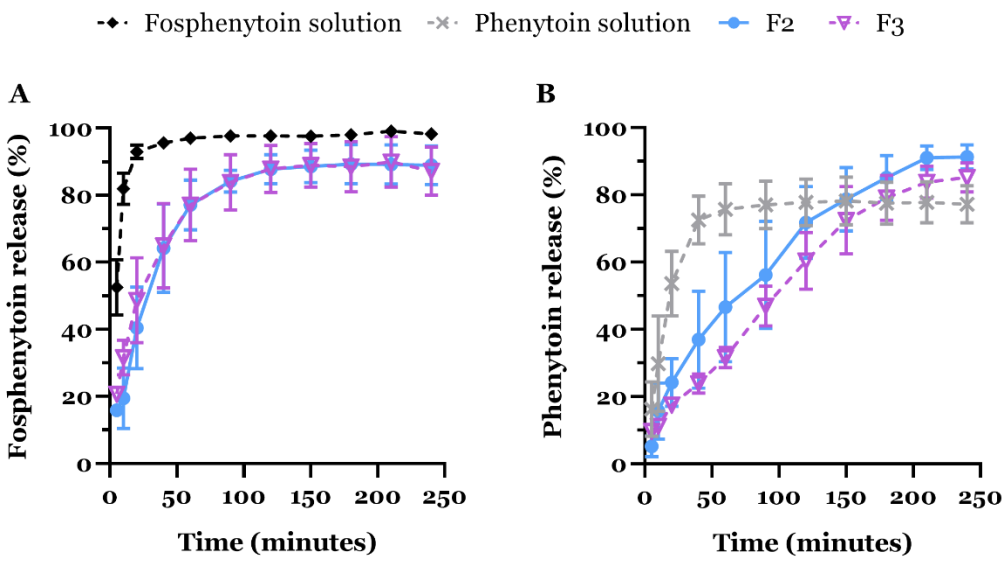

Figure 5.4. Fosphenytoin (A) and phenytoin (B) percentual drug release between 5 and 240 minutes.

Table 5.3. Fosphenytoin and phenytoin percentual drug release rate, 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 ($Y = Y/0.64$). Significance matrix is shown for the difference between formulations (slopes' comparison using an F test).

Formulation	Fosphenytoin				Phenytoin			
	Percentual drug release		Significance matrix		Percentual drug release		Significance matrix	
	R^2	Drug release rate (%·cm^{-2}·min$^{-1/2}$)	F2	F3	R^2	Drug release rate (%·cm^{-2}·min$^{-1/2}$)	F2	F3
Fosphenytoin solution*	0.8786	27.27 ± 10.14	NS	NS	-	-	-	-
Phenytoin solution**	-	-	-	-	0.9843	21.85 ± 1.95	< 0.0001	0.0016
F2	0.9868	18.55 ± 1.24	-	NS	0.9955	11.19 ± 0.31	-	NS
F3	0.9918	15.99 ± 0.84	-	-	0.9587	9.76 ± 0.83	-	-

NS – not significant (statistical difference); R^2 – linear regression's coefficient of determination; * fosphenytoin aqueous solution (42.5 mg/g); ** phenytoin (pure) Transcutol solution (5 mg/g).

Fosphenytoin's release (Figure 5.4 A and Table 5.3) was likely faster in the solution than in the microemulsions, but it was so fast that the few points fitting the linear zone in the release profile were not sufficient to calculate its release rate with confidence. Therefore,

due to this technical limitation, the differences were not statistically significant. However, this tendency accompanies the known effect that a formulation with a higher viscosity tends to slow down drug diffusion, giving it a more sustained release profile. F2 and F3 still released fosphenytoin reasonably fast and almost completely, at similar rates, and thus the characteristics of the formulations did not hinder fosphenytoin's release in a substantial way.

Phenytoin's release from the solution was not complete, reaching only about 80%, despite stabilizing at early time points (Figure 5.4B). Nevertheless, its release rate was still, as expected, significantly higher than the microemulsions' (Table 5.3). Phenytoin's release was slower in the emulsions, but not significantly different between F2 and F3, reaching up to 80-90% like with fosphenytoin, meaning that it being internalized in the formulation's droplets did not cause an increased overall retention of the drug, it only slowed it down. Also, contrary to what the results from the phenytoin solution and our previous study seemed to indicate (Chapter 4), the fact that the drug release is similar between the microemulsions seems to indicate that Transcutol did not to interfere with either fosphenytoin nor phenytoin's release in this type of formulation.

Furthermore, in general, the release from the developed microemulsions was faster than from the previously developed nanoemulsions (Chapter 4), since the release rate from the nanoemulsion that had a higher oil-to-water proportion (40:60, which was the most similar proportion to the one we chose for this study, 50:50) was two to three times lower than that of these microemulsions, for both phenytoin and fosphenytoin. This happened despite the microemulsion's viscosity being two to three times higher than said nanoemulsion. In the case of fosphenytoin, this could be due to the fact that fosphenytoin's strength was higher in the microemulsions (almost double). As for phenytoin, its release rate being higher in the microemulsions might also be due to higher strength (although the difference is not substantial, since there was 5 mg/g of phenytoin in the microemulsions and 4 mg/g of phenytoin in the mentioned nanoemulsion), but it also might be due to the microemulsion's droplets being smaller and passing through the assay membrane's pores faster and more easily.

5.3.3. *In vivo* pharmacokinetic study

Since F2 and F3 microemulsions had similar drug strengths and release profiles, we selected F3 to proceed to the *in vivo* pharmacokinetic studies, since it did not have any cosolvent and had a lower hydrophilic surfactant amount, being potentially safer. In addition to F3 (containing phenytoin and fosphenytoin) we also administered a

modification of this microemulsion, F3-FOS, a formulation having the exact same composition but without phenytoin (with fosphenytoin only), to clarify whether there is an actual need for the existence of a small amount of phenytoin (the active and permeable form) in the emulsions in order to increase and/or accelerate brain targeting, or if having fosphenytoin (the soluble and quickly bioconvertible prodrug) could be enough. The administered dose was 5.8 mg/kg of phenytoin equivalents for both groups, with the administration volume being adapted according to the drug strength within each formulation.

Similarly to the previous study, it was not possible to quantify fosphenytoin in neither brain nor blood, only phenytoin, due to a fast prodrug bioconversion, happening either before or after absorption. The obtained phenytoin's brain and blood pharmacokinetic profiles are shown in Figure 5.5. For comparison purposes, the profiles corresponding to an intravenous fosphenytoin solution (IV solution) and the fosphenytoin formulation that produced higher brain levels in the previous study (containing HPMC and albumin, IN H0.5FOS + albumin, Chapter 3) are also shown.

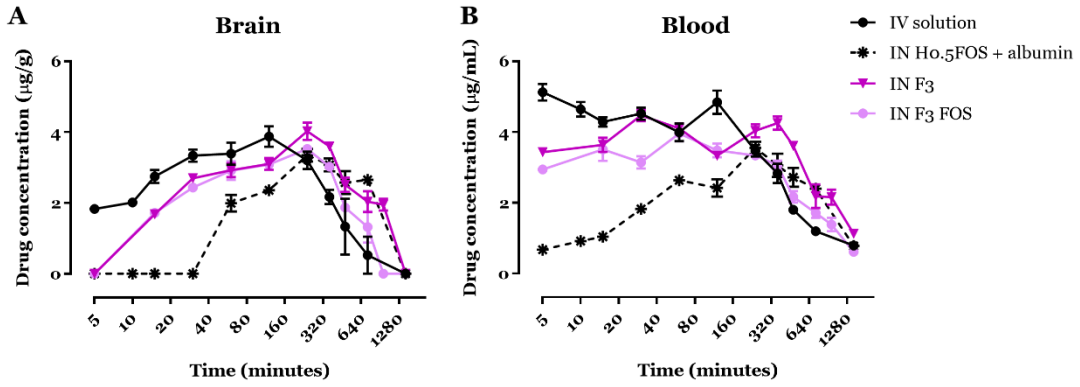

Figure 5.5. Curves of phenytoin concentration as a function of time in brain (A) and blood (B) after intranasal administration of a microemulsion containing phenytoin and fosphenytoin (IN F3) and a microemulsion containing fosphenytoin only (IN F3-FOS), compared with data produced by the same research team and previously shown (Chapter 3) – the best intranasal fosphenytoin formulation (IN H0.5FOS + albumin) and an intravenous fosphenytoin solution (IV solution). Data are presented as mean ± SEM. X axis is shown with a base 2 logarithmic transformation in order to see initial drug levels more clearly. FOS – fosphenytoin; H – hydroxypropyl methylcellulose; IN – intranasal; IV – intravenous; SEM – standard error of the mean.

Pharmacokinetic parameters and ratios were also calculated for the selected microemulsions, and are shown in Table 5.4. Just like in the previous study, given the larger uncertainty associated with phenytoin's pharmacokinetic profile in the brain obtained with the intravenous administration, we decided not to calculate brain targeting ratios that used intravenous AUC_{brain} values. Nevertheless, since blood bioavailability is

for phenytoin a good indicator of brain bioavailability as well, we calculated absolute blood bioavailability (comparison to the intravenous route), as well as brain ratios that did not use intravenous AUC_{brain} values (comparison to intranasal solution and best formulation from the previous study).

Table 5.4. Pharmacokinetic parameters determined for phenytoin levels, in both brain and blood, for microemulsions F3 and F3-FOS, both administered intranasally.

Formulation	**F3**		**F3-FOS**	
Matrix	**Brain**	**Blood**	**Brain**	**Blood**
C_{max} (µg/g or µg/mL)	4.02	4.47	3.52	3.96
T_{max} (min)	240	30	240	60
$t_{1/2el}$ (min)	653	617	322	526
k_{el} (1/min)	0.0011	0.0011	0.0022	0.0013
MRT (min)	1041	925	536	750
AUC_{0-t} (µg.min/g or µg.min/mL)	2587	3870	1770	2844
AUC_{0-inf} (µg.min/g or µg.min/mL)	4426	4872	2382	3310
AUC_{extrap} (%)	41.56	20.56	25.70	14.10
$B\%_{blood\ IN/IV}$	141		103	
$RB\%_{blood}$	153		113	
$RB\%_{brain}$	218		149	
$RB\%_{blood\ best}$	124		91	
$RB\%_{brain\ best}$	137		94	

AUC_{0-t} – area under the "drug concentration vs time" curve, from time zero to the last quantifiable drug concentration; AUC_{0-inf} – area under the "drug concentration vs time" curve, from time zero to infinity; AUC_{extrap} (%) – percentage of the area under the "drug concentration vs time" curve that was extrapolated, from the last quantifiable drug concentration to infinity; $B\%_{blood\ IN/IV}$ – absolute blood bioavailability (intranasal formulation vs intravenous solution); C_{max} – maximum drug concentration; FOS – fosphenytoin; k_{el} – elimination rate constant; MRT – mean residence time; $RB\%_{blood}$ – relative blood bioavailability (intranasal emulsion vs intranasal solution); $RB\%_{brain}$ – relative brain bioavailability (intranasal emulsion vs intranasal solution); $RB\%_{blood\ best}$ – relative blood bioavailability (intranasal emulsion vs best intranasal formulation from previous study); $RB\%_{brain\ best}$ – relative brain bioavailability (intranasal emulsion vs best intranasal formulation from previous study); $t_{1/2el}$ – elimination half-life; T_{max} – time to reach maximum drug concentration.

F3 and F3-FOS brain pharmacokinetic profiles overlapped at shorter time points, also having the same T_{max} value, which indicates that having a small amount of phenytoin (and not fosphenytoin only) did not make it faster for the drug to reach the brain. Nevertheless, brain (and blood) drug levels were consistently higher with F3 than with F3-FOS ($p < 0.001$ for brain and $p < 0.0001$ for blood) at later time points, from the T_{max} (4 h) until the end of the study (24 h) ($p < 0.05$ for brain at 6 h, and $p < 0.05$ to 0.01 for blood at 30 min and 6, 8 and 24 h). This also led to higher brain and blood AUC values with F3 when compared to the other microemulsion. These results suggest that having a

small amount of phenytoin in the active and permeable form, in addition to the soluble and quickly bioconvertible prodrug fosphenytoin, leads to an overall higher drug exposure.

The brain C_{max} of F3 was similar to the brain C_{max} of the IV solution (4.02 μg/g for F3, 3.88 μg/g for the IV solution), although taking longer to achieve (higher T_{max}). Moreover, F3 had a high absolute blood bioavailability (141%), which suggests that its intranasal administration led to better results than the intravenous group, except when concerning time to achieve maximum brain drug concentration. The microemulsion containing fosphenytoin only, F3-FOS, though, had a lower absolute blood bioavailability value (103%) than F3, but suggesting a similar performance when compared to the IV solution. This indicates that the importance of having phenytoin (plus fosphenytoin) in the formulation is reflected in absolute bioavailability, associated with improved brain and blood drug levels at later time points (from 4 h on). A higher brain and blood drug residence is also shown by looking at the MRT and $t_{1/2el}$ values, which when compared to the intravenous group were higher for both intranasal microemulsions in the brain and higher for F3 (and similar for F3-FOS) in the blood (Table 5.4).

As for comparing the results of the developed microemulsions with the intranasal simple fosphenytoin solution from the previous study, both F3 and F3-FOS had an at least moderately high relative bioavailability in brain and blood, which show their superiority. When compared to the fosphenytoin formulation (with HPMC and albumin) that had the best results in the previous study, IN H0.5FOS + albumin, both microemulsions produced higher brain and blood levels at shorter time points ($p < 0.01$ to 0.0001 for brain at 15 and 30 min, and $p < 0.05$ to 0.0001 from 5 to 60 min), which seems to suggest that the excipients that were part of the microemulsion's composition (namely the surfactants) did indeed have the expected and known drug permeation enhancing effect [151]. Nevertheless, in what concerns AUC values, overall microemulsion F3-FOS did not perform better than IN H0.5FOS + albumin, since the ratios comparing F3-FOS to that formulation were below 100%. However, F3 had a $RB\%_{blood\ best}$ value of 124% and a $RB\%_{brain\ best}$ value of 137%, which show its superiority when compared to the best fosphenytoin formulation from the previous study.

Furthermore, although it is difficult to know for sure the contribution of each component, it is likely that the initial increased brain and blood drug concentrations obtained with the microemulsions compared to the simple fosphenytoin solution are a result of an increase in the permeability of fosphenytoin solubilized in the external phase of the emulsion, either reaching the brain directly (through neuronal transport) or

indirectly (through the systemic circulation). But since brain and blood drug levels appear to increase simultaneously, and apparently proportionally, the indirect route seems to be here the most likely scenario, at least for most of the drug that reaches the brain. The higher brain C_{max} obtained with F3 (when compared to F3- FOS) seems to be due to the presence of phenytoin, that being internalized in the oil droplets could take more time to reach the brain than fosphenytoin, which is supported by it having a slower release, as described in section 5.3.2.

Hence, if there is a need for both faster and prolonged therapeutic effect, an emulsion combining both the active drug and prodrug forms would be the chosen option, since the prodrug (fosphenytoin) and the surfactants lead to higher drug levels at shorter time points (when compared to a fosphenytoin solution or fosphenytoin polymeric formulation), and the active drug (phenytoin) leads to a prolonged effect (given it can be shown to be safe). Nevertheless, if a faster effect is all that is needed, with no interest in prolonging drug exposure, a formulation promoting the fast permeation of fosphenytoin would be good enough.

5.4. Conclusion

In the previous study (Chapter 3) we had already obtained a higher phenytoin strength than any of the former reported works by using phenytoin's hydrophilic prodrug fosphenytoin (around 34.8 mg/g of phenytoin equivalents) [143–146]. In this study, by using a drug/prodrug combination, formulated in the form of an aqueous microemulsion, we were able to achieve a similar drug strength, but the absorption was faster and the obtained brain and blood drug levels were higher than those reached with intranasally administered formulations containing fosphenytoin only. Therefore, in this work we again showed that the use of phosphate ester prodrugs can be an efficient way of increasing the intranasal delivery of poorly soluble drugs such as phenytoin, with the existence of a small amount of the active and liposoluble form (formulated as microemulsion) increasing and prolonging drug levels. If there is a need for a faster and prolonged therapeutic effect, a drug-prodrug combination is ideal, but if a faster effect is all that is needed, having the prodrug alone could be enough, while considering a formulation with permeation enhancing components.

Concluding remarks

One of the main purposes of the work presented in this thesis was to prove that phosphate ester prodrugs can be an efficient strategy to increase formulation drug strength of poorly aqueous soluble drugs and, consequently, their bioavailability, namely in brain drug delivery through intranasal administration. To do so we chose an already existing low solubility drug/phosphate ester prodrug pair (phenytoin/fosphenytoin).

Since phenytoin has a very low solubility in water, and does not solubilize well in lipids either, without the use of fosphenytoin it would not have been possible to reach high drug strengths. Hence, it was not possible to obtain formulations containing phenytoin only, and even in nanosystems with a lipidic component, it was necessary to include the prodrug in order to increase drug strength.

Formulations containing only fosphenytoin (without phenytoin) already led to reasonably high brain phenytoin levels, with a formulation containing HPMC and albumin leading to a high absolute bioavailability. But even though the systematic review regarding intranasal delivery of small molecular weight drugs within nanosystems failed to discriminate the overall superiority of a nanosystem class in relation to another, in general the use of nanocarriers led to higher brain bioavailability than the respective drug solution. Hence, we developed oil-in-water nano or microemulsions which allowed the existence of a small amount of phenytoin, in the active and diffusible form, in the formulation. The selected microemulsion's *in vivo* pharmacokinetic results suggested that the existence of phenytoin, in addition to fosphenytoin, increased and prolonged brain and blood phenytoin levels, when compared with the same microemulsion containing fosphenytoin only (without phenytoin), and also when compared with the more simple intranasal fosphenytoin formulations. The importance of the permeation enhancing effects of the microemulsion was also shown, for early time points, since both tested microemulsions (the one containing only fosphenytoin, and the one containg both drugs) had increased brain and blood drug levels at shorter times.

Hence, having a phosphate ester prodrug + permeation enhancer(s) combination seems to be ideal when a faster effect is all that is necessary (without the need for prolonged drug levels). If a faster and prolonged effect is wanted, then drug + prodrug + permeation enhancers + albumin appears to be the best choice (within the studied conditions).

Nevertheless, while the simpler fosphenytoin formulations were predictably safe (safe excipients), being isotonic to slightly hypertonic, the emulsions have potentially toxic excipients in their composition, also having very high osmolality values. Even though the

selected microemulsion did not include cosolvents, which had the highest potential for toxicity, the surfactants that were part of it could also be potentially harmful. Hence, in future studies one could try to reduce the preconcentrate-to-water phase proportion in the emulsions and assess which is the lowest preconcentrate amount that results in the enhanced brain drug levels at shorter time points that were seen with the intranasal administration of the selected microemulsions. Moreover, one could try to eliminate the surfactants altogether and replace them with other permeation enhancers that could be potentially safer.

This work allowed to confirm the initial hypothesis that the use of phosphate ester prodrugs, such as fosphenytoin, can be an efficient strategy to increase the intranasal bioavailability of poorly soluble drugs, such as phenytoin, without the conversion from prodrug to parent drug delaying the drug reaching the brain (fast bioconversion, at least in the used animal model). Moreover, albumin seems to play a crucial role in prolonging brain drug exposure, as does the existence of a small amount of the active and diffusible form of the drug. Furthermore, the use of microemulsions increases drug levels at early time points, making it faster for the drug to reach the brain.

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Supplementary data

S1. Chapter 3 – Supplementary methods and results

S1.1. Supplementary methods

S1.1.1. *In vitro* drug release spectrophotometric assay validation

The calibration standards were prepared by dilution of a more concentrated simple aqueous fosphenytoin solution, using nasal simulant buffer, to obtain drug concentrations of 4.11, 8.22, 16.44, 32.88, 65.75 and 105.20 μg/mL. The lower limit of quantification (LLOQ) was defined as the lowest analyte concentration that could be quantified with acceptable inter/intraday precision and accuracy. To assess linearity, a mean calibration curve was determined using data from 6 individual curves (n = 6), each done on a different day. Precision's specification corresponded to a coefficient of variation (CV) lower than or equal to 15% (or 20% for the LLOQ) and accuracy (% of bias) had to be within ± 15% (or ± 20% for the LLOQ).

S1.1.2. *In vitro* drug release high-performance liquid chromatography assay validation

For the preparation of the calibration standards a stock solution of fosphenytoin at 5 mg/mL was made by dissolving the powder in methanol, and from this solution an intermediate one at 0.5 mg/mL was prepared by dilution in nasal fluid simulant buffer. To obtain the desired concentrations, the final dilution was also made in that same buffer, preparing calibration standards with fosphenytoin at 0.25 (LLOQ), 0.50, 2.00, 5.00, 10.00, 15.00, 20.00 and 25.00 μg/mL. Then 100 μL of each of these calibration standards were mixed with 20 μL of perchloric acid at 10% (v/v) by vortexing for 15 seconds, followed by centrifugation (microcentrifuge, Gyrozen, Daejeon, South Korea) at 13500 rpm for 5 minutes. The supernatant was then transferred into a glass vial for quantification. Quality control (QC) samples were prepared using the same methodology, but from an independent stock solution, at 0.75, 12.50 and 22.50 μg/mL (QC_1, QC_2 and QC_3, respectively). An additional sample was also prepared at the concentration of the LLOQ (QC_{LLOQ}).

S1.1.3. *In vivo* pharmacokinetic study high-performance liquid chromatography assay validation

Individual fosphenytoin and phenytoin intermediate solutions (at 0.1 mg/mL) were prepared by dilution from methanol stock solutions (at 5 mg/mL). Finally, for the spiking of the calibration standards, we prepared combined solutions of fosphenytoin and phenytoin, from either stock or intermediate solutions, by dilution in water-methanol (50:50, v/v). Fosphenytoin's concentration in these solutions was 1.5, 3.0, 10.0, 25.0, 50.0 or 75.0 μg/mL, and phenytoin's concentration was 1.5, 3.0, 10.0, 50.0, 100.0 or 150.0 μg/mL. A separate spiking solution was prepared for the internal standard, ketoprofen, at 50 μg/mL.

The calibration standards were prepared by spiking aliquots of 80 μL of blank matrices (either mice acidified blood or brain homogenate) with 20 μL of one of the combined spiking solutions (5-fold dilution), being that the final concentration ranges were 0.3 - 15 μg/mL for fosphenytoin and 0.3 - 30 μg/mL for phenytoin. QC samples were prepared at 0.9 (QC_1), 7.5 (QC_2) and 13.5 (QC_3) μg/mL for fosphenytoin and 0.9 (QC_1), 15.0 (QC_2) and 27.0 (QC_3) μg/mL for phenytoin.

After spiking, both calibration standard samples and QC samples were processed as described in Chapter 3 (section 3.2.5.2). For linearity assessment the data was analyzed according to a previously developed mathematical method [152], being transformed by a weighted linear regression, with the functions used as weighting factors being $1/x^2$ for blood samples and $1/y^2$ for brain homogenate samples. Method selectivity was evaluated by processing and analyzing blank acidified blood or brain homogenate samples (matrix without analytes or internal standard) from six different mice.

Short and long-term sample stability was also evaluated, for QC_1 and QC_3 samples, in replicate (n = 5). For the evaluation of preprocessing stability, and attempting to simulate predicted handling and storage settings, studied conditions included room temperature for 4 hours, 4 ºC for 24 hours, and -20 ºC for 10 and 30 days. Post-processing stability was evaluated at room temperature for 24 hours, which is the estimated maximum amount of time for which samples are kept in the autosampler before analysis. Additionally, the effect of 24 hour freeze-thaw cycles in unprocessed samples was also assessed by keeping them at -20 ºC and doing 3 cycles of sample unfreezing/refreezing, on three consecutive days.

S1.2. Supplementary results

S1.2.1. Gelation temperatures

Table S1.1. Gelation temperatures of formulations containing Pluronic at 14, 15 or 16% (w/w), with or without HPMC or fosphenytoin. $T_{gel}50$ was determined by applying a non-linear regression model (*log(agonist) vs. response, variable slope, four parameters*) to the "viscosity vs temperature" data. T_aMax and T_aMin were correspondingly calculated as the maximum and minimum of the second derivative of the function given by the non-linear regression model obtained for the determination of $T_{gel}50$.

Formulation	T_aMax	$T_{gel}50$	T_aMin
P14H0.2	36.7	38.4 ± 0.7	40.1
P15	32.7	34.5 ± 0.1	36.3
P15FOS	31.4	34.0 ± 0.1	36.6
P15H0.2	29.9	31.8 ± 0.1	33.6
P16	28.4	30.4 ± 0.1	32.5
P16H0.2	26.9	28.7 ± 0.1	30.4

FOS – fosphenytoin; H or HPMC – hydroxypropyl methylcellulose; P – Pluronic; T_aMax – maximum acceleration temperature, considered as the temperature at which gelation starts; T_aMin – minimum acceleration temperature, considered as the temperature at which gelation ends; $T_{gel}50$ – half-gelation temperature.

S1.2.2. *In vitro* drug release spectrophotometric assay validation

Linearity ranged from 4.11 to 105.20 μg/mL ($R^2 \geq 0.99$). The LLOQ was experimentally determined and set at 4.11 μg/mL, with adequate precision and accuracy (CV and |bias| < 17%), and all the other calibration curves' samples also showed precision and accuracy within the acceptance criteria (CV and |bias| < 10%) (Table S1.2).

Table S1.2. Precision and accuracy obtained for the calibration curves' samples in the spectrophotometric method developed to quantify *in vitro* drug release.

Nominal concentration (μg/mL)	Precision (CV, %)	Accuracy (bias, %)
4.11	17.07	9.42
8.22	9.91	1.41
16.44	7.59	2.46
32.88	8.84	2.55
65.75	2.52	-5.55
105.20	6.04	1.87

Bias – deviation from nominal value; CV – coefficient of variation; n = 6 for all studied concentration levels.

S1.2.3. *In vitro* drug release high-performance liquid chromatography assay validation

The typically obtained retention time for fosphenytoin was of approximately 4 minutes. Method selectivity was assessed by the analysis of blank samples (nasal fluid simulant buffer), which confirmed the absence of endogenous interferences at the retention time of the analyte of interest. The same occurred for the formulation vehicle (HPMC 0.5% + albumin 2%), which also had no interference at that retention time. Linearity ranged from 0.25 to 25.00 μg/mL (R^2 = 0.9991) using a weighted linear regression analysis (previously developed mathematical method [152]) and a weighting factor of $1/x^2$ (lowest relative error). The LLOQ was experimentally determined and set at 0.25 μg/mL, with adequate precision and accuracy (CV and |bias| < 5%), and the QC samples also showed precision and accuracy within the acceptance criteria (CV and |bias| < 6%), in intra and interday evaluations (Table S1.3).

Table S1.3. Intra and interday precision and accuracy obtained for the QC samples (QC_{LLOQ}, QC_1, QC_2 and QC_3) in the HPLC method developed to quantify *in vitro* drug release.

Analyte	Matrix	Nominal concentration (μg/mL)	Intraday precision (CV, %)	Intraday accuracy (bias, %)	Interday precision (CV, %)	Interday accuracy (bias, %)
Fosphenytoin	**Nasal simulant buffer**	**0.25**	4.66	-4.71	3.44	0.29
		0.75	3.48	3.25	3.93	5.16
		12.50	0.59	1.99	0.20	0.82
		22.50	0.41	3.15	0.19	2.45

Bias – deviation from nominal value; CV – coefficient of variation; HPLC – high-performance liquid chromatography; QC – quality control; for each concentration level, for interday evaluations n = 3 and for intraday evaluations n = 5.

Absolute recovery of fosphenytoin, determined for 3 concentration levels (QC_1, QC_2 and QC_3), was between 97 and 99%, with all values having an associated CV of less than 6% (Table S1.4).

Table S1.4. Absolute recovery of fosphenytoin determined for 3 different QC samples (QC_1, QC_2 and QC_3) in the HPLC method developed to quantify *in vitro* drug release.

Analyte	Matrix	Nominal concentration (μg/mL)	Absolute recovery (%)	CV (%)
Fosphenytoin	**Nasal fluid simulant buffer**	**0.75**	98.71 ± 5.44	5.51
		12.50	98.31 ± 0.49	0.50
		22.50	97.29 ± 0.45	0.47

CV – coefficient of variation; HPLC – high-performance liquid chromatography; QC – quality control; absolute recovery values are presented as mean ± standard deviation; n = 5 for each concentration level.

S1.2.4. *In vivo* pharmacokinetic study high-performance liquid chromatography assay validation

The analysis of six blank mice acidified blood or brain homogenate samples (each from a different animal) confirmed the absence of endogenous interferences at the retention times of the analytes: 4 minutes for fosphenytoin, 9 - 10 minutes for phenytoin (both at 215 nm) and 15 - 16 minutes for the internal standard (at 280 nm) (Figures S1.1 to S1.4).

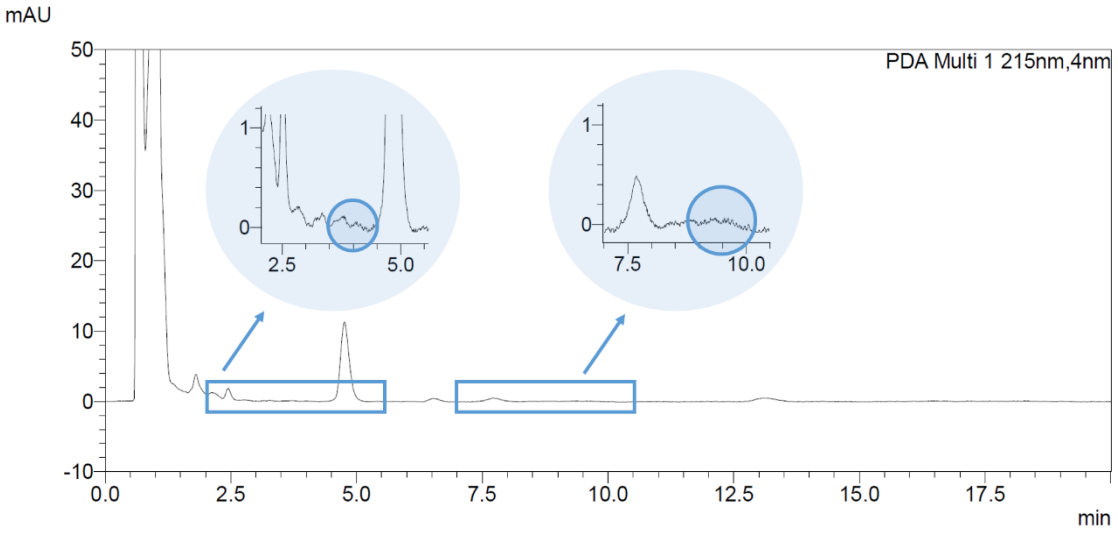

Figure S1.1. Chromatogram of a blank mouse acidified blood sample, with detection at 215 nm.

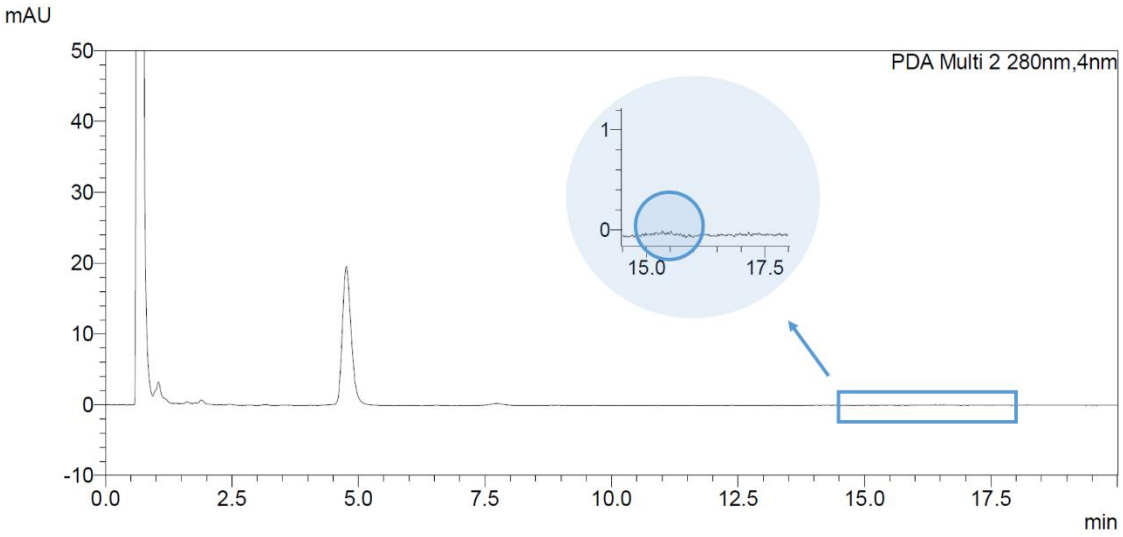

Figure S1.2. Chromatogram of a blank mouse acidified blood sample, with detection at 280 nm.

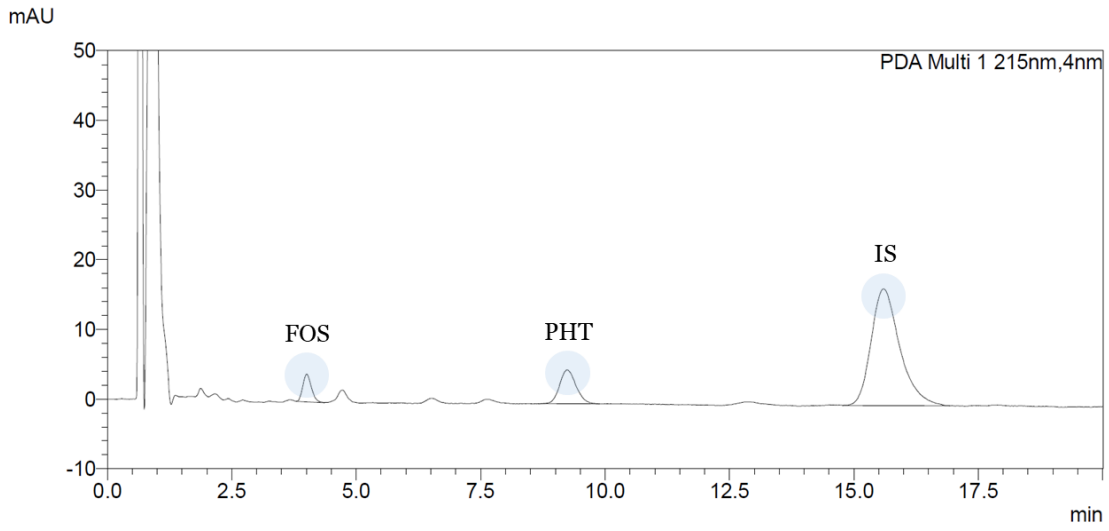

Figure S1.3. Chromatogram of a spiked mouse acidified blood sample, concentration of 2 µg/mL for fosphenytoin and phenytoin, and 10 µg/mL for the IS, with detection at 215 nm; FOS – fosphenytoin; IS – internal standard; PHT – phenytoin.

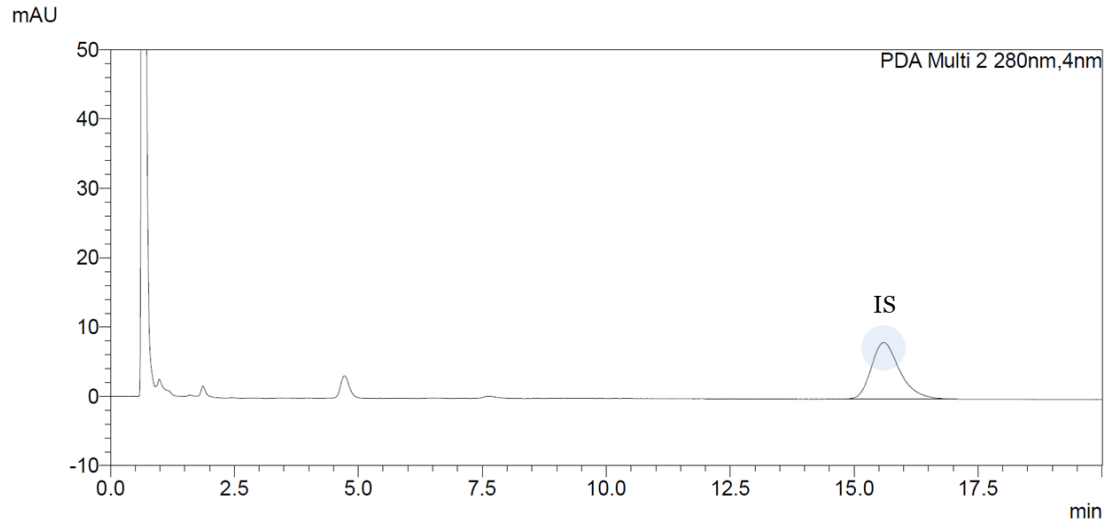

Figure S1.4. Chromatogram of a spiked mouse acidified blood sample, concentration of 2 µg/mL for fosphenytoin and phenytoin (not visible), and 10 µg/mL for the IS, with detection at 280 nm; IS – internal standard.

Linearity was observed at 0.3 - 15.0 µg/mL for fosphenytoin and 0.3 - 30.0 µg/mL for phenytoin, in both acidified blood and brain homogenate samples, and for all individual and mean calibration curves ($R^2 \geq 0.99$), using a weighted linear regression analysis (previously developed mathematical method [152]) to correct heteroscedasticity.

The LLOQ's were experimentally determined and set at 0.3 μg/mL, with adequate precision and accuracy (CV and |bias| < 12%) for both matrices (Table S1.5). However, in the brain this corresponds to approximately 1.5 μg/g, since 4 mL of diluted acid were added to 1 gram of tissue, to produce the brain homogenate used as matrix. The QC samples also showed precision and accuracy within the acceptance criteria (CV and |bias| < 14%), in intra and interday evaluations.

Table S1.5. Intra and interday precision and accuracy obtained for the QC samples (QC_{LLOQ}, QC_1, QC_2 and QC_3) for fosphenytoin and phenytoin, in blood and brain.

Analyte	Matrix	Nominal concentration (μg/mL)	Intraday precision (CV, %)	Intraday accuracy (bias, %)	Interday precision (CV, %)	Interday accuracy (bias, %)
Fosphenytoin	**Blood**	**0.3**	2.95	-1.30	10.88	8.32
		0.9	7.55	-5.59	10.04	-1.26
		7.5	1.43	-11.52	12.00	-3.09
		13.5	6.68	-8.75	12.15	6.54
	Brain	**0.3**	1.45	10.45	3.84	1.88
		0.9	5.34	5.07	3.06	0.70
		7.5	4.65	-6.07	4.88	-5.43
		13.5	3.21	-4.62	0.55	-4.40
Phenytoin	**Blood**	**0.3**	2.29	-11.86	8.14	-2.57
		0.9	1.44	-12.76	3.59	-13.40
		15.0	4.96	2.72	5.74	6.28
		27.0	2.75	-9.72	8.23	-7.81
	Brain	**0.3**	3.59	-5.40	2.37	-7.59
		0.9	5.26	-2.61	5.74	-4.22
		15.0	5.26	-7.89	4.98	-8.88
		27.0	1.71	-7.58	1.26	-7.33

Bias – deviation from nominal value; CV – coefficient of variation; QC – quality control; for each concentration level (per analyte and matrix type), for interday evaluations n = 3 and for intraday evaluations n = 5.

Absolute recovery was between 50 - 53% and 74 - 79% for fosphenytoin, and 78 - 81% and 85 - 89% for phenytoin, in blood and brain, respectively. Absolute recovery of the IS was also evaluated and was between 73 - 74% in both matrices. All values had an associated CV of less than 11% (Table S1.6).

Table S1.6. Absolute recovery of fosphenytoin and phenytoin, determined for 3 different QC samples (QC_1, QC_2 and QC_3), in blood and brain. Values for the IS are also shown.

Analyte	Matrix	Nominal concentration (µg/mL)	Absolute recovery (%)	CV (%)
Fosphenytoin	**Blood**	**0.9**	52.57 ± 5.28	10.04
		7.5	49.68 ± 2.80	5.64
		13.5	49.94 ± 5.44	10.89
	Brain	**0.9**	77.36 ± 5.04	6.51
		7.5	74.14 ± 4.97	6.71
		13.5	78.63 ± 2.36	3.00
Phenytoin	**Blood**	**0.9**	81.11 ± 4.65	5.74
		15.0	78.23 ± 1.93	2.46
		27.0	77.51 ± 2.97	3.84
	Brain	**0.9**	89.14 ± 5.86	6.57
		15.0	84.74 ± 3.74	4.41
		27.0	86.29 ± 1.22	1.41
IS	**Blood**	**10.0**	73.36 ± 3.64	4.97
	Brain		73.72 ± 3.28	4.45

CV – coefficient of variation; IS – internal standard; QC – quality control; absolute recovery values are presented as mean ± standard deviation; n = 5 for each concentration level (per analyte and matrix type), except for the IS (n = 15, for each matrix).

Fosphenytoin and phenytoin were stable, before processing, at room temperature for 4 hours, at 4 ºC for 24 hours, and at -20 ºC for 10 and 30 days, in both matrices (stability condition/reference ratio between 89 and 115% for fosphenytoin, and between 85 and 114% for phenytoin). Samples were also stable after processing while having been kept at room temperature for 24 hours (stability condition/reference ratio between 86 and 106% for fosphenytoin, and between 103 and 114% for phenytoin). Moreover, 3 freeze-thaw cycles in 3 consecutive days showed no substantial sample degradation (stability condition/reference ratio between 96 and 101% for fosphenytoin, and between 94 and 99% for phenytoin). See Table S1.7 for further detail.

Table S1.7. Stability of fosphenytoin and phenytoin at variable time and temperature conditions, determined for 2 different QC samples (QC_1 and QC_3), in blood and brain.

Analyte	Matrix	Nominal concentration (µg/mL)	Processed sample	Unprocessed sample				
			RT, 24h	RT, 4h	4 ºC, 24h	-20 ºC, 10 days	-20 ºC, 30 days	UR cycles
FOS	**Blood**	**0.9**	88.2	101.1	104.1	112.5	113.8	100.9
		13.5	86.0	105.1	103.6	110.5	114.8	98.4
	Brain	**0.9**	93.1	97.5	94.1	88.9	111.1	99.6
		13.5	106.0	100.8	104.7	98.4	107.1	95.6
PHT	**Blood**	**0.9**	111.4	94.7	94.8	111.2	111.5	98.8
		27.0	113.9	100.1	93.3	107.0	107.7	96.1
	Brain	**0.9**	103.4	97.0	106.0	85.0	107.4	94.0
		27.0	106.2	100.3	103.7	94.6	113.6	98.8

FOS – fosphenytoin; PHT – phenytoin; QC – quality control; RT – room temperature; UR – unfreezing/refreezing; n = 5 for each concentration level (per analyte and matrix type).

S2. Chapter 4 – Supplementary methods and results

S2.1. Supplementary methods

S2.1.1. *In vitro* drug release high-performance liquid chromatography assay validation

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, containing 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 LLOQ was the lowest analyte concentration that could be quantified with inter/intraday precision and accuracy within the acceptance criteria. 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 LLOQ (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 standard 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 [152], 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 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 autosampler 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.

S2.2. Supplementary results

S2.2.1. *In vitro* drug release high-performance liquid chromatography assay validation

The validated HPLC method was based on the one developed for the quantification of fosphenytoin formulations' drug release samples (Chapter 3, section 3.2.4.2, and

supplementary data, section S1.1.2). 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%) 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. Typical mean retention times were of approximately 4 minutes for fosphenytoin and 10 minutes for phenytoin (example chromatogram in Figure S2.1).

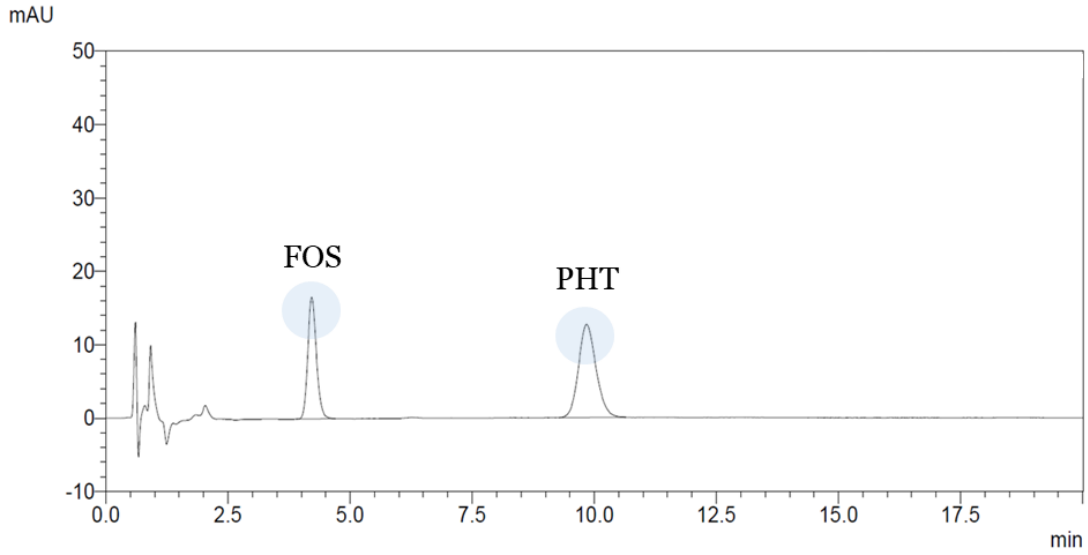

Figure S2.1. Chromatogram of a calibration standard, concentration of 5 µg/mL for both fosphenytoin and phenytoin, with detection at 215 nm; FOS – fosphenytoin; PHT – phenytoin.

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).

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 S2.1).

Table S2.1. Intra and interday precision and accuracy obtained for the QC samples (QC_{LLOQ}, QC_1, QC_2 and QC_3) for fosphenytoin and phenytoin.

Analyte	Nominal concentration (µg/mL)	Intraday precision (CV, %)	Intraday accuracy (bias, %)	Interday precision (CV, %)	Interday accuracy (bias, %)
Fosphenytoin	**0.1**	4.60	7.46	5.30	2.24
	0.3	3.25	0.36	1.87	2.62
	20.0	0.19	-3.77	0.24	-3.62
	36.0	0.13	-1.44	0.27	-1.34
Phenytoin	**0.1**	3.65	4.08	7.48	7.99
	0.3	3.48	4.14	4.36	2.77
	20.0	0.17	-0.75	0.11	-2.69
	36.0	0.58	0.25	1.96	-2.38

Bias – deviation from nominal value; CV – coefficient of variation; QC – quality control; n = 5 for all studied concentration levels.

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 S2.2).

Table S2.2. Absolute recovery of fosphenytoin and phenytoin, determined for 3 different QC samples (QC_1, QC_2 and QC_3).

Analyte	Nominal concentration (µg/mL)	Absolute recovery (%)	CV (%)
Fosphenytoin	**0.3**	100.96 ± 3.77	3.74
	20.0	93.92 ± 0.27	0.29
	36.0	95.62 ± 0.12	0.12
Phenytoin	**0.3**	99.72 ± 4.38	4.39
	20.0	95.57 ± 0.19	0.20
	36.0	96.61 ± 0.50	0.52

CV – coefficient of variation; QC – quality control; absolute recovery values are presented as mean ± standard deviation; n = 3 for each concentration level.

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 S2.3).

Table S2.3. Stability of fosphenytoin and phenytoin at variable time and temperature conditions, determined for 2 different QC samples (QC_1 and QC_3).

Analyte	Nominal concentration (µg/mL)	24 hours, room temperature	Three 24 hour freeze/thaw cycles
Fosphenytoin	**0.3**	101.5	101.5
	36.0	99.4	99.7
Phenytoin	**0.3**	102.2	102.4
	36.0	112.9	113.3

QC – quality control; n = 5 for each concentration level.