1 **Title**

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

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Abstract

Intranasal administration could increase both safety and efficacy of drugs acting on the central nervous system, but low solubility severely limits administration through this route. Phenytoin's prodrug, fosphenytoin, is hydrophilic and freely soluble in water, but less permeable since it is dianionic. We aimed to assess whether this phosphoester prodrug could be a suitable alternative to phenytoin in intranasal delivery. Secondly, we aimed to compare simple formulation strategies in fosphenytoin delivery.

Fosphenytoin formulations containing thermosensitive and/or mucoadhesive (hydroxypropyl methylcellulose, HPMC) polymers were developed, guided by viscosity, gelling temperatures, osmolality, and *in vitro* drug release tests. Then, a pharmacokinetic study was performed, comparing an intravenous fosphenytoin solution, an intranasal fosphenytoin solution, and intranasal fosphenytoin mucoadhesive formulations with or without albumin.

Formulations containing HPMC allowed high drug strengths, and had a relatively fast release profile, which was not changed by albumin. Intranasal administration of a formulation with HPMC and albumin prolonged drug concentration over time and led to complete or even increased absolute bioavailability. Moreover, phenytoin's blood levels did not reach the high peak obtained with intravenous administration. In conclusion, the use of phosphate ester prodrugs could be an efficient and safe strategy to increase the intranasal bioavailability of poorly soluble drugs.

Keywords: Albumin, Brain delivery, Epilepsy, Fosphenytoin, Intranasal, Pharmacokinetics.

Abbreviations:

AUC – area under the drug concentration vs time curve; $AUC_{0\text{-}t}$ – AUC from time zero to the last quantifiable drug concentration; $AUC_{0\text{-}inf}$ – AUC from time zero to infinite; AUC_{extrap} (%) – percentage of the AUC that was extrapolated, from the last quantifiable drug concentration to infinite; AUMC – area under the first moment curve; F – absolute blood bioavailability (intranasal formulation vs intravenous solution); C_{last} – last quantifiable drug concentration; C_{max} – maximum drug concentration; FDA – Food and Drug Administration; FOS – fosphenytoin; HPLC – high-performance liquid chromatography; H or HPMC – hydroxypropyl methylcellulose; IN – intranasal; IV – intravenous; k_{el} – elimination rate constant; MRT – mean residence time; P – Pluronic F-127; R^2 – coefficient of determination; $RB\%_{blood}$ – relative blood bioavailability (intranasal formulation vs intranasal solution); SEM – standard error of the mean; $t_{1/2el}$ – elimination half-life; $T_{gel}50$ – half-gelation temperature; T_{max} – time to reach maximum drug concentration.

1. Introduction

Epilepsy is a high incidence chronic neurological disorder, affecting millions of people worldwide (World Health Organization, 2019). It is characterized by the occurrence of epileptic seizures, which happen when there is a disturbance in the normal electrical activity of the brain, with an excessively synchronous or sustained neuronal discharge (Musumeci et al., 2019). There are several types of seizures with many different clinical manifestations, including partial or total impairment of consciousness, that may or may not be accompanied by repeated and/or unusual body movements (Kiriakopoulos and Shafer, 2017). Convulsive *status epilepticus* is the most severe of all epileptic seizures (Glauser et al., 2016). In the hospital setting, first-line treatment of convulsive *status epilepticus* is usually a benzodiazepine, specifically lorazepam (intravenous), diazepam (intravenous) or midazolam (intramuscular) (Glauser et al., 2016). Second-line treatment includes the intravenous administration of several other antiseizure drugs. 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 (Glauser et al., 2016; Poplawska et al., 2015; Shih et al., 2016; Zaccara et al., 2017). Nevertheless, intravenous administration requires hospitalization, trained personnel, and establishment of intravenous access, which can delay therapeutic response. Furthermore, it is an invasive route of administration, which makes it uncomfortable and even painful for the patients, with risk of injury at the administration site (Kapoor et al., 2016).

The intranasal route could be an alternative to parenteral anticonvulsive drug administration due to several associated advantages, being a more pleasant and patient-friendly option, and also having proven to be overall faster in achieving seizure cessation, since there is no need for hospitalization, being easy to administer by a caregiver (Glauser et al., 2016; Kapoor et al., 2016; U.S. Food and Drug Administration, 2019; Zaccara et al., 2017). Furthermore, intranasal delivery allows direct nose-to-brain transport, which means that at least part of the drug will bypass the blood-brain barrier, and can get to the brain directly, hence reducing systemic distribution and potentially increasing safety (Erdó et al., 2018; Pires and Santos, 2018). Intranasal administration of antiepileptics, namely midazolam and diazepam, has shown to be as effective as the intravenous alternatives, having become first-line options in pre-hospital setting (off-label use), and with one preparation having even reached the market (Nayzilam®, a midazolam formulation) (U.S. Food and Drug Administration, 2019). However, benzodiazepines' use may cause somnolence, and drug formulations required to solubilize benzodiazepines have been shown to induce lacrimation and nose and throat irritation (Maglalang et al., 2018). Moreover, if abused, they can also cause deleterious cognitive effects and dependence/tolerance.

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* (Antunes Viegas et al., 2016). 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.

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 the composition of nasal formulations, alone or in combination (Karavasili and Fatouros, 2016; Shaikh et al., 2011). The addition of albumin to the formulation was also evaluated, since fosphenytoin strongly binds to it, and albumin has been described to be actively transported from the nasal cavity to the brain (Falcone et al., 2014; Lai et al., 1995). 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.

2. Materials and methods

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).

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 (%).

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. 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.

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).

Experimental protocol was adapted from a previously developed method (Pires et al., 2020). The bottom chamber was filled with 1.8 mL of nasal fluid simulant buffer, pH 6.5 (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). 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 2.4.1. and 2.4.2. A simple fosphenytoin aqueous solution was used as positive control.

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 (FDA et al., 2018), 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 appendix A (section A.1).

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.* (Antunes Viegas et al., 2016). 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 (FDA et al., 2018). Method selectivity was also evaluated for the formulation vehicle. Further detailed information can be found in the appendix A, (section A.2).

2.5. *In vivo* pharmacokinetic study

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.

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 K_3 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 re-extracted 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 (FDA et al., 2018), 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 appendix A (section A.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 ($X = \text{Log}_{10} X$ for Pluronic + HPMC formulations at 32 ºC). $T_{gel}50$, the half-gelation temperature, 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 an adaptation of the Higuchi model (Ramteke et al., 2014; United States Pharmacopeial Convention, 2017), 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) (Zhang et al., 2010). 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 (C_{max}) in blood and brain, and the corresponding 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-t}) was calculated through the linear trapezoidal method. The area under the drug concentration vs time curve, from time zero to infinite (AUC_{0-inf}) was calculated by adding AUC_{0-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 $AUC_{0-inf} = AUC_{0-t} + C_{last}/k_{el}$. 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_{0-t} (the formula being $MRT = AUMC/AUC_{0-t}$). The percentage of the AUC that was extrapolated, from the last quantifiable drug concentration to infinite, was also calculated (AUC_{extrap} (%)).

Absolute blood bioavailability (F) of the intranasal formulations was calculated with equation 1:

$$F = \frac{AUC_{blood_{IN}}}{AUC_{blood_{IV}}} \times 100 \quad (1).$$

Relative blood bioavailability ($RB\%_{blood}$) was used to compare intranasally administered formulations to a simple intranasal aqueous drug solution, and it was calculated by equation 2:

$$RB\%_{blood} = \frac{(AUC\ _{blood_{IN}})_{formulation}}{(AUC\ _{blood_{IN}})_{solution}} \times 100 \qquad (2).$$

3. Results and discussion

3.1. Formulation development with rheology and osmolality characterization

Osmolality measurements and rheological studies were used to support the decision making on 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 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.

1 **Table 1.** Viscosity and osmolality of vehicles containing HPMC only, Pluronic only, or mixtures of HPMC
2 and Pluronic. Data correspond to 1 representative batch for each different vehicle. Osmolality data are
3 presented as mean ± SEM. Viscosity was measured at 20 ºC, and in Newtonian fluids corresponds to the value
4 at the highest torque. Viscosity of non-Newtonian fluids is represented as zero shear viscosity, inferred by
5 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

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

8

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

16 As for the combination of the two polymers, it was only possible to obtain physically stable
17 vehicles with HPMC at 0.2%, since with HPMC at 0.5 or 1% phase separation occurred after some
18 time (varying between a few hours to a few days, sooner for higher polymer concentrations), and
19 for the highest polymer concentrations a precipitate appeared. This physical instability in vehicles
20 containing Pluronic and HPMC in combination (at higher polymer concentrations) has not, to the
21 best of our knowledge, been previously reported in the scientific literature, even while having a
22 wide variety of studies using them. Hence, we decided on combining Pluronic (at various
23 concentrations) with HPMC at 0.2% only. The addition of HPMC to Pluronic at 16% changed its
24 rheological behavior from Newtonian to non-Newtonian (pseudoplastic) at 20 ºC (not shown).

25 Pluronic undergoes temperature induced sol-gel transitions. If the polymer's concentration is not
26 high enough, it transitions from a low viscous fluid to a more viscous one and not to a solid gel, but
27 we will still refer to it as gelation. With dispersions of Pluronic alone, gelation occurred at 15 and
28 16%, while for polymer concentrations equal or below 14% the viscosity only slightly increased
29 with temperature increase up to 45 ºC (Figure 1A and appendix B, section B.1). The combination of
30 the two polymers slightly increased the viscosity compared to Pluronic alone at 20 ºC (Table 1),
31 but, more substantially, it also anticipated Pluronic's gelation (Figure 1A and appendix B, section
32 B.1). Moreover, with Pluronic at 14% in combination with HPMC a transition to increased viscosity
33 did in fact occur.

1

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

9

10 At 32 ºC, both HPMC and Pluronic showed non-Newtonian pseudoplastic behavior (Figures 1B and
11 1C). For Pluronic at 16% it was not possible to evaluate viscosity over a wide shear velocity range,
12 since at lower rotational speeds the torque was too high, with a corresponding viscosity above the
13 spindle's measurement range. Furthermore, the addition of 0.2% HPMC to Pluronic resulted in
14 increased zero shear viscosity at 32 ºC (Figure 1D).

15 In what concerns drug incorporation, for vehicles containing HPMC only the concentration of 1%
16 was excluded because it led to a high zero shear viscosity at room temperature, which could
17 hindrance administration, especially through nasal instillation. Therefore, we selected the
18 concentrations of 0.2 and 0.5%. Drug incorporation into these vehicles increased zero shear
19 viscosity at both studied temperatures, but not substantially (for the highest concentration, 0.5%, it
20 only increased about 3 cP at 20 ºC and about 1 cP at 32 ºC) (Figure 2A and Tables 1 and 2). For
21 vehicles containing Pluronic only, polymer concentration of 16% was excluded due to its gelation
22 temperature being too low ($T_{gel}50$ 30.4 ºC), having the risk of undergoing sol-gel transition at an
23 increased room temperature (on a hot summer day, for example), which could also make it difficult
24 to administer. Hence, concentrations from 12 to 15% were selected, and drug addition to these
25 vehicles increased zero shear viscosity considerably, at both studied temperatures, but more
26 substantially at 32 ºC (for the highest concentration, 15%, it increased about 26 cP at 20 ºC and
27 about 122621 cP at 32 ºC) (Figure 2A and Tables 1 and 2). Moreover, there was also an effect of
28 drug addition on gelation, which was anticipated, hence occurring at lower temperatures, with
29 Pluronic at 15% plus fosphenytoin having a sol-gel transition near the mean nasal temperature
30 (Figure 2B and appendix B, section B.1).

31

32 **Figure 2**. Zero shear viscosity at 32 ºC of vehicles (clear pattern columns) and drug formulations (striped
33 pattern columns), determined by non-linear regression (A); and viscosity variation with temperature increase
34 at a constant shear rate (100 s^{-1}) for Pluronic vehicle (continuous connecting line) and Pluronic drug
35 formulation (discontinuous connecting line) (B); 1 to 3 batches for each formulation. Data are presented as
36 mean ± SEM. FOS – fosphenytoin; H or HPMC – hydroxypropyl methylcellulose; P – Pluronic; SEM –
37 standard error of the mean.

38

39

Table 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 – 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 2A and Tables 1 and 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 2). Although hypertonic, these last formulations were still within the established limits for marketed nasal preparations (Marx et al., 2015), 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 (Falcone et al., 2014). 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).

3.2. *In vitro* drug release

The viscosity of a formulation may, on the one hand, increase its retention in the nasal cavity, thereby also 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 appendix B (sections B.2 and B.3).

In what concerns percentual drug release (Figure 3) and percentual drug release rate (Table 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. Fosphenytoin's percentual drug release between 5 and 180 minutes. FOS – fosphenytoin; H - hydroxypropyl methylcellulose; P – Pluronic.

Table 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 ± 0.2	-	-	-	-	-	NS
P13H0.2FOS	0.9981	9.8 ± 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, administered 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 (Marx et al., 2015).

3.3. *In vivo* pharmacokinetic study

Bioanalytical method validation results are presented in the appendix B (section B.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 (Antunes Viegas et al., 2016). 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. 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 (Muchohi et al., 2002; U.S. Food and Drug Administration, 2011a; Walton et al., 1999). 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.

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

9

10 **Figure 4.** Curves of phenytoin concentration as a function of time in brain (A) and blood (B). Data are
11 presented as mean ± SEM. Only significance levels for significant differences obtained when comparing one
12 condition to all others are shown: **** $p < 0.0001$ and ** $p < 0.01$, two-way ANOVA analysis with Tukey's
13 multiple comparisons post-test; FOS – fosphenytoin; H – hydroxypropyl methylcellulose; IN – intranasal; IV
14 – intravenous.

15

16 **Table 4.** Pharmacokinetic parameters determined for phenytoin levels, in both brain and blood, for all tested
17 formulations and administration routes.

Formulation	Fosphenytoin solution				H0.5FOS		H0.5FOS + albumin	
Administration route	Intravenous		Intranasal		Intranasal		Intranasal	
Matrix	Brain	Blood	Brain	Blood	Brain	Blood	Brain	Blood
C_{max} (µg/g or µg/mL)	3.88	5.13	3.32	3.59	3.22	3.44	3.35	3.55
T_{max} (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
F	-		-	92	-	83	-	113
RB% $_{blood}$	-		-	-	-	91	-	123

18 AUC_{0-t} – area under the curve of drug concentration as a function of time, from time zero to the last quantifiable drug
19 concentration; AUC_{0-inf} – area under the curve, from time zero to infinite; AUC_{extrap} (%) – percentage of the AUC that was
20 extrapolated, from the last quantifiable drug concentration to infinite; F – absolute blood bioavailability (intranasal
21 formulation vs intravenous solution); C_{max} – maximum drug concentration; FOS – fosphenytoin; H – hydroxypropyl
22 methylcellulose; IN – intranasal; IV – intravenous; k_{el} – elimination rate constant; MRT – mean retention time; RB% $_{blood}$
23 – relative blood bioavailability (intranasal formulation vs intranasal solution); $t_{1/2el}$ – elimination half-life; T_{max} – time to
24 reach maximum drug concentration.

25

26 The brain and blood profiles of the intranasal drug solution and the intranasal H0.5FOS formulation
27 largely overlap (Figure 4). Therefore, overall HPMC did not benefit nor reduce drug absorption.
28 Phenytoin's C_{max} in the brain was relatively similar between all formulations and administration
29 routes (3.2 to 3.9 µg/g) (Table 4). Blood C_{max} was also quite similar for all intranasal formulations
30 (3.4 to 3.6 µg/mL) but was substantially higher for the intravenous administration (5.1 µg/mL),
31 which suggests that the intranasal route could be safer in what concerns maximum systemic drug

levels. Importantly, this small single dose administration already achieved half of the lower limit of these rodents' therapeutic level, which is around 7-12 μg/mL (Markowitz et al., 2010).

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 4). This could, hypothetically, be explained by intracellular neural transport to the brain, as previously demonstrated for albumin (Falcone et al., 2014), since intracellular transport is slow when compared to extracellular diffusion, and/or increased retention in the nasal cavity.

Given the larger uncertainty obtained in brain phenytoin's pharmacokinetic profile in intravenous administration, partially due to the high limit of quantification of the method, elimination and AUC parameters are shown (Table 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 30% higher than the simple aqueous intranasal fosphenytoin solution (Table 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 (National Center for Biotechnology Information, n.d.). 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 (U.S. Food and Drug Administration, 2011b). 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 (Baharvand et al., 2014; Teo et al., 2016). Self-emulsifying drug delivery systems for oral delivery reported phenytoin concentrations reaching up to 25 mg/mL (Atef and Belmonte, 2008; Ye et al., 2019). 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 35 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.

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.

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5. Declaration of interests

The authors declare no conflicts of interest.

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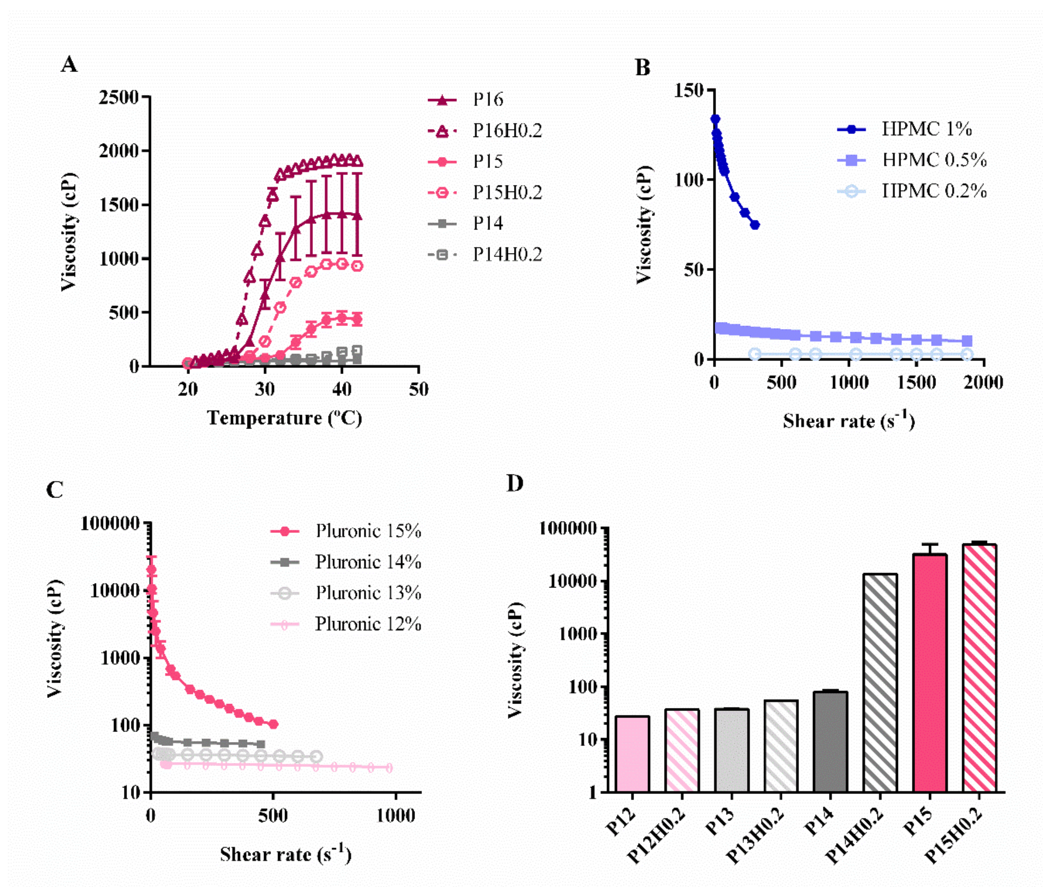
1 # Figures



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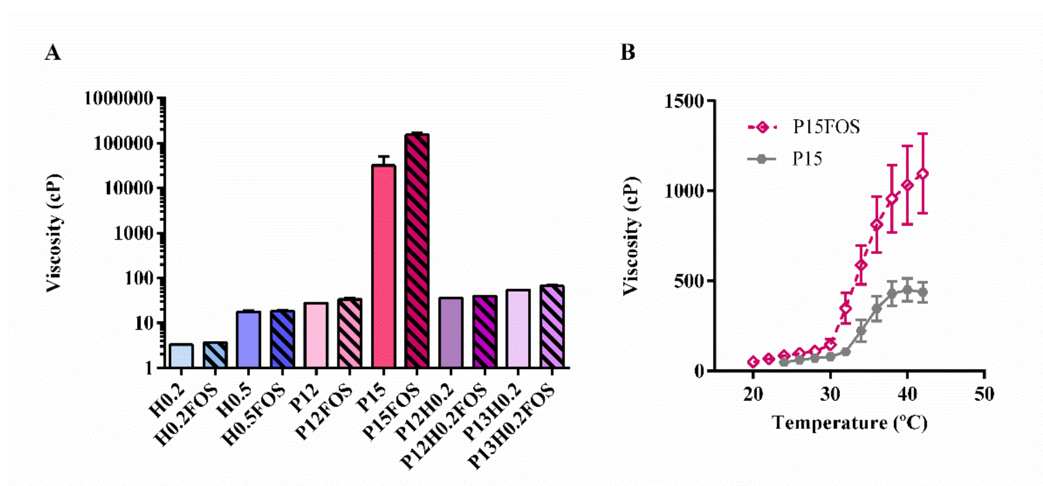
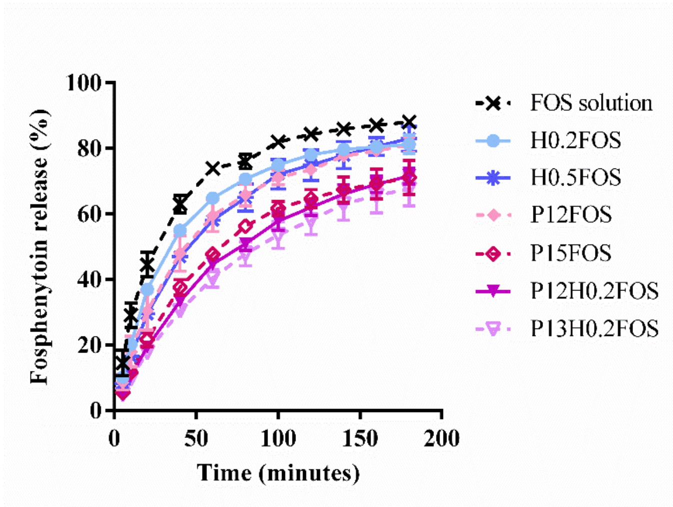
3 **Figure 1.**



4

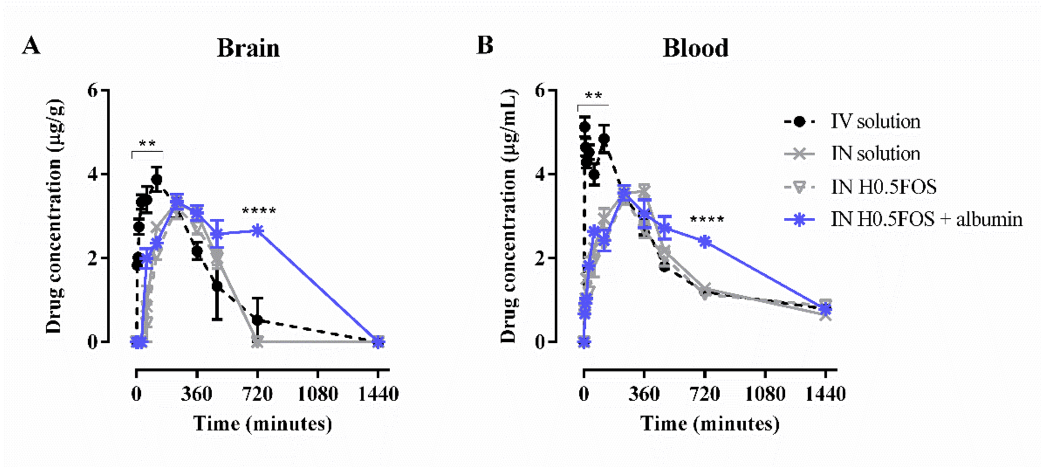
5 **Figure 2.**

1



2 **Figure 3.**

3



4 **Figure 4.**

5

Appendices

Appendix A. Supplementary methods

A.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 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).

A.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}).

A.3. *In vivo* pharmacokinetic study high-performance liquid chromatography assay validation

From methanol stock fosphenytoin and phenytoin solutions intermediate ones at 100 µg/mL were prepared by dilution. 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, 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 section 2.5.2 of the main article. For linearity assessment the functions used as weighting factors in the transformation of the data by a weighted linear regression were $1/x^2$ for blood samples and $1/y^2$ for brain samples. Method selectivity was evaluated by processing and analyzing blank acidified blood or brain samples (matrix without analytes or internal standard) from six different mice.

Short and long-term sample stability were also evaluated for QC_1 and QC_3 samples, in replicates (n = 5). For the evaluation of pre-processing 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 auto-sampler 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.

Appendix B. Supplementary results

B.1. Gelation temperatures

Table B1. 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;

B.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 B2).

Table B2. 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.

B.3. *In vitro* drug release high-performance liquid chromatography assay validation

The validated high-performance liquid chromatography method was adapted from the one developed by Antunes Viegas *et al.*, as described in the main article's materials and methods section [12]. 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 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 B3).

Table B3. Intra and interday precision and accuracy obtained for the quality control samples (QC_{LLOQ}, QC_1, QC_2 and QC_3) in the high-performance liquid chromatography 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; 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 B4).

Table B4. Absolute recovery of fosphenytoin determined for 3 different quality control samples (QC_1, QC_2 and QC_3) in the high-performance liquid chromatography 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; absolute recovery values are presented as mean ± standard deviation; n = 5 for each concentration level.

B.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) (Figure B1, A to D).

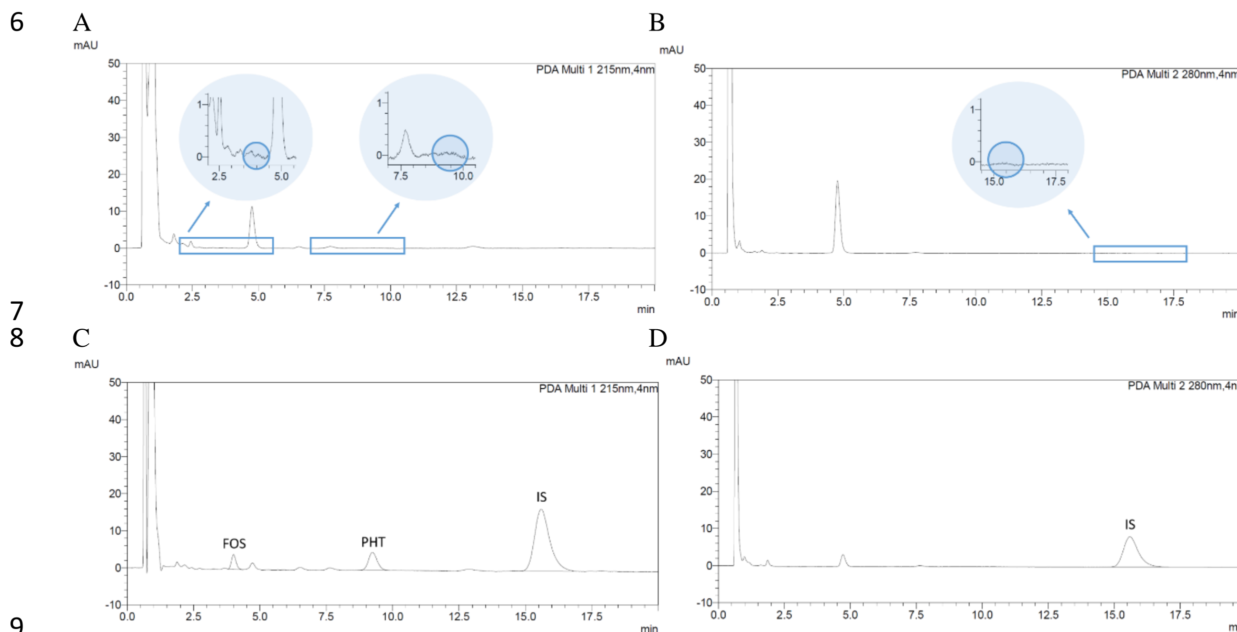


Figure B1. Example chromatograms. A - Blank mouse blood sample, detection at 215 nm. B - Blank mouse blood sample, detection at 280 nm.C. C. Spiked mouse blood sample, concentration of 2 µg/mL for fosphenytoin and phenytoin, and 10 µg/mL for the internal standard, with detection at 215 nm; D. Spiked mouse blood sample, concentration of 2 µg/mL for fosphenytoin and phenytoin (not visible), and 10 µg/mL for the internal standard, with detection at 280 nm. FOS – fosphenytoin; IS – internal standard; PHT – phenytoin.

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 to correct heteroscedasticity. The resulting mean calibration curves' equations and corresponding coefficients of determination can be seen in Table B5.

Table B5. Calibration range and mean calibration curves' parameters for fosphenytoin and phenytoin, in both blood and brain.

Analyte	Matrix	Calibration range (µg/mL)	Mean calibration curve equation	R^2
Fosphenytoin	Blood	0.3 - 15.0	y = 0.0796x - 0.0042	0.9934
	Brain homogenate		y = 0.1281x - 0.0068	0.9922
Phenytoin	Blood	0.3 - 30.0	y = 0.1922x - 0.0024	0.9929
	Brain homogenate		y = 0.2179x - 0.0026	0.9964

R^2 – coefficient of determination; y represents analyte/IS peak ratio; x represents analyte concentration (µg/mL); mean equations determined from multiple values for each concentration level (n = 3, in 3 different days); IS – internal standard.

1 The LLOQ's were experimentally determined and set at 0.3 µg/mL, with adequate precision and
2 accuracy (CV and |bias| < 12%) for both matrices (Table B6). However, in the brain this corresponds
3 to approximately 1.5 µg/g, since 4 mL of diluted acid were added to 1 gram of tissue, to produce the
4 brain homogenate used as matrix. The QC samples also showed precision and accuracy within the
5 acceptance criteria (CV and |bias| < 14%), in intra and interday evaluations.

6

7 **Table B6**. Intra and interday precision and accuracy obtained for the quality control samples (QC_{LLOQ}, QC_1,
8 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

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

11

12 Absolute recovery was between 50 - 53% and 74 - 79% for fosphenytoin, and 78 - 81% and 85 - 89%
13 for phenytoin, in blood and brain, respectively. Absolute recovery of the internal standard was also
14 evaluated and was between 73 - 74% in both matrices. All values had an associated CV of less than
15 11% (Table B7).

16 **Table B7**. Absolute recovery of fosphenytoin and phenytoin, determined for 3 different quality control samples
17 (QC_1, QC_2 and QC_3), in blood and brain. Values for the internal standard 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
Internal standard	Blood	10.0	73.36 ± 3.64	4.97
	Brain		73.72 ± 3.28	4.45

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

3

4 Fosphenytoin and phenytoin were stable, before processing, at room temperature for 4 hours, at 4 ºC
5 for 24 hours, and at -20 ºC for 10 and 30 days, in both matrices (stability condition/reference ratio
6 between 89 and 115% for fosphenytoin, and between 85 and 114% for phenytoin). Samples were also
7 stable after processing while having been kept at room temperature for 24 hours (stability
8 condition/reference ratio between 86 and 106% for fosphenytoin, and between 103 and 114% for
9 phenytoin). Moreover, 3 freeze-thaw cycles in 3 consecutive days showed no substantial sample
10 degradation (stability condition/reference ratio between 96 and 101% for fosphenytoin, and between
11 94 and 99% for phenytoin). See Table B8 for further detail.

12

13 **Table B8**. Stability of fosphenytoin and phenytoin at variable time and temperature conditions, determined for
14 2 different quality control 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

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

17