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RESEARCH ARTICLE

A sensitive LC-MS/MS method for quantification of phenytoin and its major metabolite with application to in vivo investigations of intravenous and intranasal phenytoin delivery

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Phenytoin is a powerful antiseizure drug with complex pharmacokinetic properties, making it an interesting model drug to use in preclinical in vivo investigations, especially with regards to formulations aiming to improve drug delivery to the brain. Moreover, it has a major metabolite, 5-(4-hydroxyphenyl)-5-phenylhydantoin, which can be simultaneously studied to achieve a better assessment of its behaviour in the body. Here, we describe the development and validation of a sensitive LCMS/MS method for quantification of phenytoin and 5-(4-hydroxyphenyl)-5-phenylhydantoin in rat plasma and brain which can be used in such preclinical studies. Calibration curves produced covered a range of 7.81 to 250 ng/mL (plasma) and 23.4 to 750 ng/g (brain tissue) for both analytes. The method was validated for specificity, sensitivity, accuracy, and precision and found to be within the acceptable limits of ±15% over this range in both tissue types. The method when applied in two in vivo investigations: validation of a seizure model and to study the behaviour of a solution of intranasally administered phenytoin as a foundation for future studies into direct nose-to-brain delivery of phenytoin using specifically developed particulate systems, was highly sensitive for detecting phenytoin and 5-(4-hydroxyphenyl)-5-phenylhydantoin in rat plasma and brain.

KEYWORDS
5-(4-hydroxyphenyl)-5-phenylhydantoin, drug delivery, intranasal, liquid chromatography–tandem mass spectrometry, phenytoin

Article Related Abbreviations: 4-HPPH, 5-(4-hydroxyphenyl)-5-phenylhydantoin; MRM, multiple reaction monitoring; TBME, tert-butyl methyl ether.

1 | INTRODUCTION

Phenytoin is a powerful, fast, and long-acting antiseizure drug useful for generalized and partial seizures, however, it is hampered by poor water solubility and pharmacokinetic complexity [1]. It undergoes hepatic metabolism primarily by CYP2C9 and CYP2C19 to produce the pharmacologically inactive and slightly water-soluble major metabolite 5-(4-hydroxyphenyl)-5-phenylhydantoin (4-HPPH), which is subsequently metabolized to the very water soluble 4-HPPH-O-glucuronide and excreted in the urine. Complexity arises because the conversion to 4-HPPH is saturable within the narrow therapeutic range, imparting a nonlinear and dose-dependent pharmacokinetic elimination profile to the drug which also exhibits significant interpatient variability [2, 3]. In addition to this, phenytoin is also a substrate for the multidrug transporter P-glycoprotein (P-gp). P-gp is highly expressed in brain capillary endothelial cells and functions as a protective efflux transporter, ejecting molecules back out into the blood plasma, restricting their passage into the brain parenchyma [4, 5]. These complex properties make phenytoin a very interesting model drug for pharmaceutical studies, as evidenced by a large number of studies available in the literature, covering a range of fields including formulation science [6, 7], pharmacokinetic characterization [8, 9], and pharmacodynamic investigations in seizure models [10, 11]. Given the complexity of phenytoin pharmacokinetics, simultaneous investigation of the concentrations of its major metabolite 4-HPPH, although not always studied, may yield important supplementary information on its distribution and metabolism in the body, especially with respect to the in vivo evaluation of novel targeted drug delivery systems, a field which is rapidly advancing in pharmaceutical science. For this reason, we saw a need to create a simple analytical assay which could be used in preclinical evaluations to maximize the information gained from such studies.

The aim of this study was to develop and validate a simple and sensitive LC MS/MS method for simultaneous determination of phenytoin and its major metabolite 4-HPPH in the plasma and brains of rats for application primarily in drug delivery studies utilizing phenytoin. To demonstrate its application, the method was first used to measure plasma and brain concentrations after a therapeutic intravenous dose of phenytoin which was administered during the validation of a seizure model in our laboratory. Subsequently, the application of its sensitivity was demonstrated by measuring brain and plasma concentrations after the intranasal administration of a phenytoin solution. This was carried out as preliminary work for investigations into the intranasal delivery of phenytoin using particulate systems which are being designed in our laboratory to potentially deliver phenytoin and its powerful antiseizure effects to the brain while bypassing its many systemic hurdles.

2 | MATERIALS AND METHODS

2.1 | Materials

Phenytoin (5,5-diphenylhydantoin) sodium injection (50 mg/mL) (DBL Phenytoin Injection BP) was purchased from Hameln Pharmaceuticals GmbH (Germany). Isotonic (0.9% w/v) saline was purchased from Baxter (Australia). Isoflurane was provided by the Hercus-Taieri Resource Unit, University of Otago. Phenytoin sodium, 4-HPPH (5-(4-Hydroxyphenyl)-5-phenylhydantoin), propylene glycol (PG), formic acid (for mass spectrometry, ~98%), and phosphate-buffered saline sachets (pH 7.4) were purchased from Sigma Aldrich (New Zealand). Deuterated phenytoin (d_{10}-phenytoin; (5,5-(diphenyl-d_{10}) hydantoin)) was purchased from Toronto Research Chemicals (Canada). All water used in this study was ion exchanged, distilled, and passed through a Milli-Q water purification system (Millipore, USA). Acetonitrile (LiChrosolv), methanol (LiChrosolv), tert-butyl methyl ether (TBME) (LiChrosolv), and ethanol (EMSURE) were purchased from Lab Supply (New Zealand). All solvents were LC grade. Male Wistar rats (~300 to 380 g) were obtained from the HTRU, University of Otago, housed under specific pathogen-free conditions, and given food and water ab libitum. The experiments were approved by the Animal Ethics Committee, University of Otago. Rat plasma and brain tissue for LC-MS/MS method validation and standard preparation was obtained from control rats administered saline treatments and collected using the same methods as the phenytoin-treated samples in this study.

2.2 | Preparation of standards

Stock solutions for standard preparation were produced by dissolving analyte powders (phenytoin, 4-HPPH, and d_{10}-phenytoin) in methanol at a concentration of 1 mg/mL. Serial dilutions of these stock solutions in methanol were carried out to produce the desired standard concentrations. Brain homogenate was prepared by homogenizing brain tissue on ice with a tip sonicator (UP50H Ultrasonic Processor, Hielscher Ultrasound Technology) after adding 2 mL/g of Milli Q water. The homogenates were stored in aliquots of 100 μL at −80°C. Plasma was thawed and used without further dilution, as the lower viscosity allowed it to be aliquoted accurately. To prepare standard

samples for analysis, aliquots of phenytoin and 4-HPPH standard solutions (5 μL) and a d_{10}-phenytoin internal standard solution (5 μL) were mixed with blank plasma or brain homogenates. Standards were prepared to cover a final concentration range (after extraction) of 7.81 to 250 ng/mL for plasma and 23.4 to 750 ng/g for brain tissue. Quality control samples were prepared with standards at concentrations within the calibration ranges.

The standard samples were vortex mixed, then 200 μL of chilled acetonitrile was added and sonicated briefly in a water bath (Elmasonic S 60 (H), Elma Ultrasonics). A 800 μL aliquot of chilled TBME was then added to the samples, which were sonicated again (typically for up to 5 s), and then vortex mixed briefly (typically for up to 5 s). Samples were then centrifuged at 17,200 G for 20 min at 4°C (Prism R Microcentrifuge, Labnet International) and moved into a precooled tray from where a 800 μL aliquot of the supernatant was transferred to a new tube and the supernatant subsequently evaporated to dryness in a centrifugal evaporator (Thermo Savant Speed Vac) (8 to 12 h). A 200 μL volume of methanol was then added to each tube and briefly sonicated and vortex mixed to reconstitute. This was followed with a short centrifugation (10,000 rpm for 1 s) to make sure all of the liquid was moved to the base of each tube. Samples were filtered through a 13 mm Nylon 0.22 μm syringe filter (Microanalytix) into a 250 μL insert (PP BM insert with bottom spring case, Phenomenex) in a 2 mL clear glass vial (Thermo Scientific). Samples were stored at ambient temperature until analysis.

2.3 | LC-MS/MS conditions

Sample analysis was conducted using an Agilent 1290 HPLC system (G4226A autosampler, LC binary SL pump, TCC SL) coupled to an AbSciex QTRAP 5500 mass spectrometer with Turbo Spray ion source run in positive mode. The optimized mobile phase A was 0.1% formic acid in Milli Q water and mobile phase B was 0.1% formic acid in 2:1 acetonitrile:methanol. The analysis used a flow rate of 0.25 mL/min, injecting 5 μL of each sample into a Kinetex EVO 5 μm 100 Å C_{18} (150 × 2.1 mm) column, preceded by a 4 × 2.0 Gemini-NX C_{18} SecurityGuard Cartridge. The column was maintained at 40°C. Starting pressure was approximately 1700 psi. The gradient began at 80% A, 20% B, where it was held for 30 s before shifting to 5% A, 95% B over 7 min to elute the analytes. It was then held at this ratio for 5 min to clean out matrix components, then returned to 80% A, 20% B over 30 s, and allowed to re-equilibrate for 6 min, giving a total run time of 19 min. Eluent was allowed to flow to the mass spectrometer detector for the first 6.9 min for compound elution, then was diverted to waste until 13 min, then allowed to flow to the detector again until 19 min to re-equilibrate. A solution of 90% methanol in Milli Q water was used to clean the needle between samples (10 s). The draw speed and eject speed were each set at 200 μL/min. The autosampler was maintained at 20°C during analysis.

2.4 | Method validation

The method validation assessed specificity, sensitivity, accuracy, and precision. Analyst software was used to collect the data. Analyte or internal standard ratio was used to plot calibration curves and analyses the data in GraphPad Prism (GraphPad Software Inc., USA). The assays were validated using triplicate samples on three separate days. The lower (LLOQ) and upper (ULOQ) limits of quantification of each of the assays was determined experimentally by analyzing accuracy and precision of standards, with limits of ±15% considered acceptable [12]. Intra- and interday variability were assessed by way of the quality control samples for which acceptable accuracy and precision limits were taken to be ±15%. Calculation of accuracy was performed by taking the values of the standards as quantified by the assay and expressing them as percentages of the nominal standard concentrations that were expected. Calculation of precision was performed using the ratio of the SD to the mean of a set of measurements. This is presented as percentage coefficient of variation (CV%), otherwise known as the relative standard deviation (RSD). Standard curves and chromatogram data were plotted in GraphPad Prism.

2.5 | Application of the LC-MS/MS method to in vivo phenytoin studies

2.5.1 | Drug administration

Animals were anesthetized with 5% isoflurane and an oxygen flow rate of 1 mL/min for 3.5 min so they were unconscious during drug administration. For the intravenous administration study, commercial phenytoin sodium solution (25 mg/kg ≈ 150 to 180 μL) was administered intravenously via a lateral tail vein using a 0.3 mL Lo-dose U-100 insulin syringe with 29 G × 12.7 mm needle (BD Biosciences). For the intranasal administration study, a 100 mg/mL phenytoin solution was prepared by dissolving phenytoin sodium in a mixture of ethanol (50% v/v), PG (10% v/v), and water (40% v/v) with a brief sonication. The solution was kept at 37°C immediately prior to administration, to maintain a solution of phenytoin with no visible precipitate. A 15 cm long piece of PE 10 tubing (Fort Richard Laboratories) was threaded onto the 29 G

needle of a 0.3 mL BD Ultrafine Lo-dose insulin syringe. Drug solution was drawn into the syringe through the tubing and adjusted to a volume of 20 μL. The tubing was then threaded into a Rat Intranasal Catheter Device (RICD) (Impel Neuropharma) in preparation for administration. Anesthetized rats were laid on the bench in a supine position. The tip of the RICD was promptly and gently positioned inside one nostril and the tubing gently guided in until 1 cm had entered the nasal cavity. At this point, the syringe was gently depressed to administer the dose. The apparatus was held in position for 5 s, following administration, then was gently pulled out of the nasal cavity. The rat was then moved to a recovery cage where it quickly regained consciousness.

2.5.2 | Tissue collection

Rats were euthanized by guillotine decapitation at approximately 65 min, following the drug administration. Trunk blood was collected in sodium heparin coated tubes (BD Biosciences) at the time of euthanasia and centrifuged at the conclusion of the experiment (2000 G for 10 min at ambient temperature (Heraeus Multifuge X3FR, Thermo Scientific) so the plasma supernatant could be collected and frozen. The brain was also dissected, rinsed in PBS, and frozen at −80°C, until required for LC-MS/MS analysis.

2.5.3 | Sample preparation

Experimental samples were prepared for LC-MS/MS analysis using the same method as described above for the calibration curve samples. A pilot run was conducted before the main study to estimate phenytoin concentrations in the tissues and where appropriate, samples were diluted with blank plasma or brain homogenate to be quantifiable within the standard range. Each 100 μL sample aliquot had 5 μL internal standard (d_{10}-phenytoin) and 5 μL methanol (standard solvent) added for consistency with the calibration curve standards.

2.5.4 | Statistical analysis

All statistical analyses were performed using GraphPad Prism. The concentration data from the tissue distribution studies were compared statistically using unpaired, two-tailed *t*-tests, or ANOVA with Tukey's post-hoc test as appropriate. For brain to plasma ratio data, a ratio-paired *t*-test was used to determine if the ratio differed statistically from 1.0. A *p*-value of <0.05 was interpreted as a statistically significant result.

3 | RESULTS AND DISCUSSION

This study aimed to develop and validate a sensitive and simple LC-MS/MS method for quantification of phenytoin and its major metabolite 4-HPPH in rat plasma and brain tissue for application to in vivo investigations. Previously reported LC-MS/MS methods for phenytoin quantification have all been developed for the application of measurement of the drug in human plasma [13–20], while in vivo studies in rat plasma or brain tissue have used the less sensitive method of HPLC [5, 9, 21-25], which our study aimed to improve on. With the exception of Tanaka et al. [24] (who measured both phenytoin and 4-HPPH in rat plasma), none of the methods found in published studies that measured at least one of the analytes in at least one of these rat tissues presented detailed validation data or a reference to a study providing this. This highlighted the need for a validated and published method that could be used in future in vivo studies employing phenytoin in rat models.

3.1 | Sample preparation

The extraction procedure used in this study was based on our previously reported method for extracting another hydrophobic compound, oleoylethanolamide, from rat plasma and brain tissue [26]. Prior to extraction, brain tissue was homogenized in water to create an evenly dispersed homogenate that could be accurately aliquoted [27], unlike after homogenization in organic solvent, as reported by Marchi et al. [23], which in preliminary experiments resulted in extensive precipitation. Phenytoin and 4-HPPH were extracted from aliquots of plasma or brain homogenate using a combination of acetonitrile and TBME (1:4). Both solvents have been used independently in other phenytoin extraction methods [5, 13, 18, 21, 28-30] as TBME is very good for liquid–liquid extraction due to its immiscibility with the aqueous phase and acetonitrile is a very effective organic or aqueous-miscible protein precipitant. However, the value of combining them has been highlighted by Xue et al. [31]. They described how the proportion of acetonitrile used could modulate the polarity of the organic solvent and influence extraction of analytes and collateral matrix components in addition to effectively precipitating proteins and preventing the formation of a protein emulsion on addition of TBME. Xue et al. [30, 31] used a ratio of approximately 1:8, but found that ratios down to 1:3 could be used without the

TABLE 1 Optimized parameters for phenytoin and 4-HPPH analysis.

Entrance potential (V)	**10.0**
Curtain gas (psi)	15.0
Collision gas	Medium
Ionspray voltage (V)	5500.0
Temperature (°C)	600.0
Ion source gas 1 (psi)	40.0
Ion source gas 2 (psi)	40.0

acetonitrile separating from the TBME phase into the aqueous phase and losing its ability to influence the polarity of the organic phase. Acetonitrile is, therefore, likely to have had a polarity modulating role in our procedure which had a ratio of 1:4, as well as the protein precipitating effect which was important for phenytoin as it is a highly protein bound drug. The other advantage to using TBME is its low density as such its organic phase sits on top during liquid–liquid extraction, simplifying the extraction compared to the likes of Tanaka et al. [24] who used a more dense solvent, dichloromethane, which formed a less accessible bottom layer.

3.2 | Optimization of LC-MS/MS conditions

The first step in optimization of the LC-MS/MS method was optimization of ionization and fragmentation of the analytes by direct infusion into the mass spectrometer, the results of which are shown in Table 1. The majority of LC-MS methods reported for measuring phenytoin have used positive ESI mode [14, 16, 18–20], while less were found to use negative mode [13, 15, 17]. Both modes were tested during our initial infusion studies, but positive mode was found to give a greater signal intensity and was used in subsequent development to promote sensitivity. Figure 1 shows the molecular structures and molecular masses of the analytes as well as the fragmentation in positive-ion mode (arrows). As predicted from the fragmentation shown in Figure 1 and previous literature [16, 18], the precursor/product ion pairs found to produce the highest intensity in positive-ion mode were 253.011/182.100 for phenytoin, 263.152/192.088 for d_{10}-phenytoin, and 269.051/198.100 for 4-HPPH. The parameters for their detection were optimised in multiple reaction monitoring (MRM) mode, as shown in Table 2.

Existing HPLC methods for phenytoin in plasma or brain have for the most part employed a range of mobile phases composed of methanol or acetonitrile (or a combination of the two), often in combination with an acidic buffer to keep phenytoin (pK_a = 8.3) in an unionized form [5, 9, 20, 22–25, 28–30, 32-34]. For LC-MS/MS, methods have most commonly used methanol and water with formic acid or ammonium acetate as ionization enhancers or pH modifiers [14-16, 18–20], so these were used as a starting point for the development of our method. While separation in plasma was acceptable, using methanol as the organic phase led to distorted peaks in brain tissue in this study indicating a less clean separation. It was determined that this could be rectified by mixing with acetonitrile and an organic phase comprising 2:1 acetonitrile:methanol was found to produce clean chromatograms in plasma and brain tissue. This mobile phase was used in another study reported by our group for the detection of oleoylethanolamide in rat plasma and brain and it was also found useful for analysis in these tissues in that case [26]. The improvement with the addition of acetonitrile is possibly due to the complex array of matrix components and the lower elution strength of methanol compared to acetonitrile in RP chromatography. Mixing a protic and aprotic organic solvent with different elutropic strengths in the organic phase offers a wider potential for solubility of the unmonitored matrix components which would also

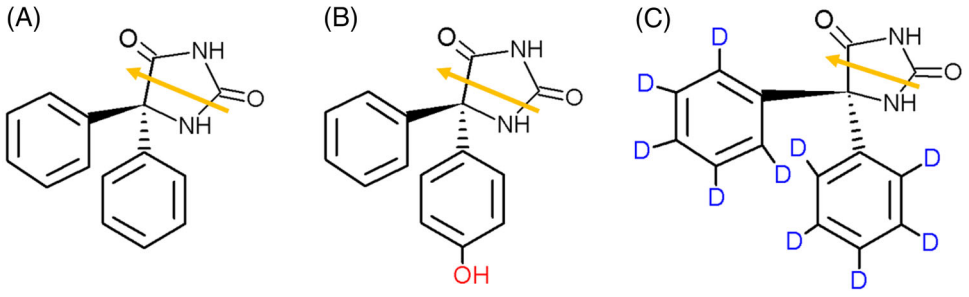

FIGURE 1 Molecular structures of (A) Phenytoin (Mw = 252.3 g/mol), (B) 4-HPPH (Mw = 268.3 g/mol), and (C) d_{10}-phenytoin (Mw = 262.3 g/mol). The expected fragmentation point which produces the predominant daughter ion of each ($[M+H]^+$) is shown with a yellow arrow

TABLE 2 MRM optimized parameters for ions monitored.

Q1	Q3	Time (ms)	ID	DP (V)	CE (V)	CXP (V)
253.011	182.100	150.0	Phenytoin	71	27	10
263.152	192.088	150.0	d_{10}-Phenytoin	31	37	12
269.051	198.100	150.0	4-HPPH	71	25	6

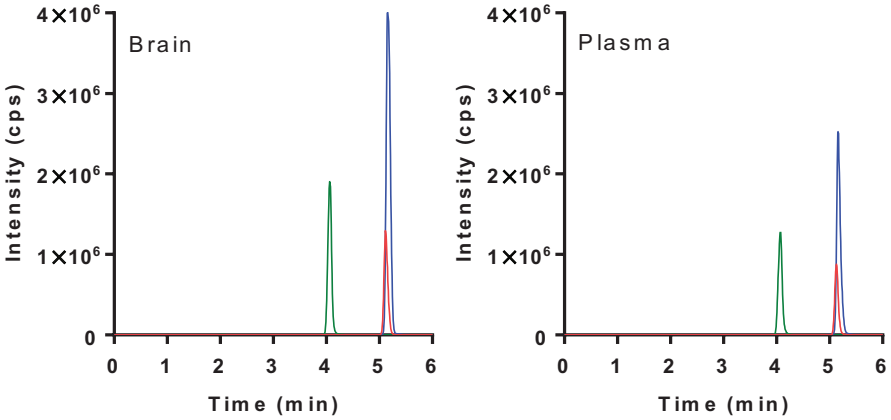

FIGURE 2 Representative chromatograms of phenytoin (blue), d_{10}-phenytoin (red), and 4-HPPH (green) extracted from rat brain homogenate (left) and rat plasma (right). The phenytoin and 4-HPPH concentrations of the analytes in the samples used to produce these chromatograms were 125 ng/mL in plasma and 375 ng/g in brain tissue (equivalent to 125 ng/mL in the diluted brain homogenate). The internal standard (d_{10}-phenytoin) concentration was equivalent to 90 ng/mL in plasma and 270 ng/g in brain tissue (equivalent to 90 ng/mL in the diluted brain homogenate). Note that original data have been plotted using GraphPad Prism to enhance clarity

have been passing through the column with the analytes and interacting with the column. As well as the improved chromatogram, the presence of acetonitrile also allowed backpressure to be decreased, permitting a higher flow rate and decreased run time so sample throughput could be higher. Finally, as mentioned above, formic acid and ammonium acetate have both been used previously as ionization enhancers for phenytoin detection [13, 15, 16, 18, 20] and were both trialled in the development of this method. It was found that formic acid increased signal intensity of the analytes in these tissues compared with ammonium acetate and it was, therefore, taken forward for use in the optimised mobile phase. The gradient of the method was adjusted by trial and error to optimize the separation of phenytoin and 4-HPPH, as presented in the chromatograms in Figure 2.

3.3 | Validation of the LC-MS/MS method

3.3.1 | Specificity

The optimised method parameters produced three clear peaks representing the two analytes and internal standard in rat plasma and brain tissue (Figure 2). Phenytoin eluted consistently at 5.16 min, 4-HPPH at 4.06 min, and the internal standard d_{10}-phenytoin at 5.12 min in samples from both matrices. Blank samples, containing neither of the analytes, confirmed the specificity of the signal.

3.3.2 | Sensitivity

To determine the sensitivity of the assay, standard curves were produced by plotting mean analyte or internal standard ratio values against concentration. The data were initially evaluated with a more commonly used linear regression model, but it was found that a quadratic ($1/x^2$) regression model provided a better fit due to a nonlinear response of the instrument at higher concentrations, as has been reported in another phenytoin LC-MS/MS method [20]. The standard curve was, therefore, validated using the quadratic model in triplicate on three separate days (Figure 3). Accuracy and precision for all concentration values was within an acceptable range of ±15% and the fit of the curve maintained an R^2 value of greater than 0.99. The LLOQ for both analytes was 7.81 ng/mL in plasma and 23.4 ng/g in brain tissue. The present method with LLOQs showed greater sensitivity compared to the method reported by Tanaka et al. [24] (measured both the analytes simultaneously) and others [16, 18, 35, 36] (measured phenytoin only), where LOQ for both the analytes was found to be as 50 ng/mL.

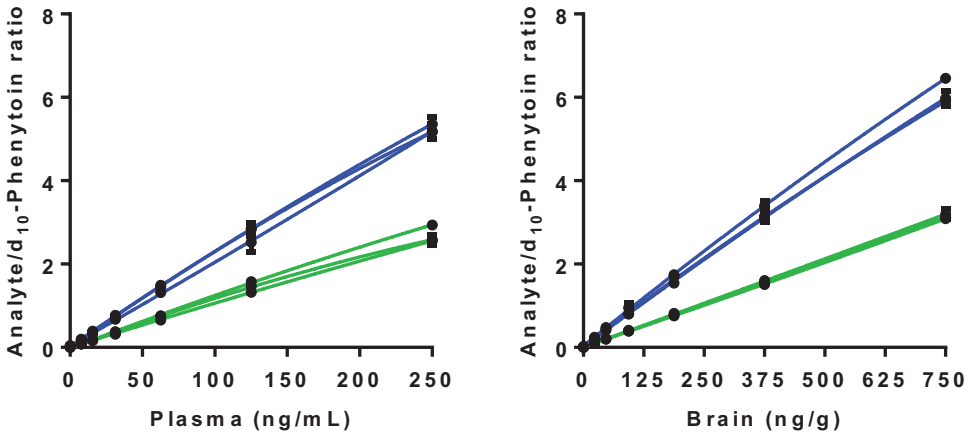

FIGURE 3 Standard curves of phenytoin (blue) and 4-HPPH (green) in plasma (left) and rat brain homogenate (right). Data shown are the mean values (±standard deviation) of the standards prepared and measured in triplicate on three different days

TABLE 3 Interday accuracy and precision of phenytoin and 4-HPPH quantification in plasma (ng/mL) and rat brain homogenate (ng/g) based on quality control samples.

Analyte in plasma	Nominal conc. (ng/mL)	Interday (n = 3) Mean (ng/mL)	Accuracy (%)	Precision (CV%)
Phenytoin	15.6	15.3	98.1	4.0
	62.5	62.2	99.6	3.1
	250	255.2	102.1	4.5
4-HPPH	15.6	16.8	107.6	4.3
	62.5	65.0	104.0	3.2
	250	272.2	108.9	3.6
Analyte in Brain	**Nominal conc. (ng/g)**	**Interday (n = 3) Mean (ng/g)**	**Accuracy (%)**	**Precision (CV%)**
Phenytoin	46.9	49.8	106.	2.8
	187.5	192.4	102.6	2.4
	750	768.8	102.5	1.6
4-HPPH	46.9	49.1	104.7	3.5
	187.5	185.9	99.2	2.1
	750	750.5	100.1	1.7

3.3.3 | Accuracy and precision

The inter- and intraday accuracy and precision of the assays, based on quality control samples, are shown in Tables 3 and 4, respectively. Variability was within an acceptable range of ±15% for all assays.

Standard curves were also constructed in brainstem and olfactory bulb homogenates, so that these brain regions could be analyzed separately from the remainder of the brain (henceforth referred to as the main brain) to provide more insight into intranasal pathways of phenytoin delivery (Figure 4). The mean values were found to be within the precision and accuracy limits (±15%) stated for the validated brain method, indicating that the standard curve of the analytes did not differ significantly between the different types of brain tissues, and samples could be compared directly.

The intraday accuracy and precision of the assays, based on quality control samples, are shown in Table 5. Variability was within the acceptable range of ±15% for both brainstem and olfactory bulb assays.

3.3.4 | Recovery and matrix effects

The recovery and matrix effects of phenytoin from plasma and brain samples were determined by comparing the peak area ratio in prespiked samples, postspiked (after extracting the blank samples phenytoin and metabolite were spiked), and neat samples (in methanol). Samples

TABLE 4 Intraday accuracy and precision of phenytoin and 4-HPPH quantification in rat plasma (ng/mL) and brain (ng/g) homogenate based on quality control samples.

		Intraday 1 (n = 3)			Intraday 2 (n = 3)			Intraday 3 (n = 3)		
Analyte in plasma	**Nominal conc. (ng/mL)**	**Mean (ng/mL)**	**Accuracy (%)**	**Precision (%CV)**	**Mean (ng/mL)**	**Accuracy (%)**	**Precision (%CV)**	**Mean (ng/mL)**	**Accuracy (%)**	**Precision (%CV)**
Phenytoin	15.6	15.8	92.6	5.1	14.5	92.6	5.1	15.75	100.8	3.0
	62.5	59.6	102.7	5.9	64.2	102.7	5.9	62.9	100.7	1.2
	250	243.8	100.4	2.9	250.9	100.4	2.9	270.9	108.4	1.1
4-HPPH	15.6	17.1	109.1	1.9	17.5	112.3	5.5	15.8	101.4	4.0
	62.5	65.2	104.4	4.5	67.4	107.8	7.3	62.3	99.7	3.5
	250	266.9	106.8	4.8	285.8	114.3	2.7	263.9	105.6	1.4
		Intraday 1 (n = 3)			**Intraday 2 (n = 3)**			**Intraday 3 (n = 3)**		
Analyte in Brain	**Nominal conc. (ng/g)**	**Mean (ng/g)**	**Accuracy (%)**	**Precision (%CV)**	**Mean (ng/g)**	**Accuracy (%)**	**Precision (%CV)**	**Mean (ng/g)**	**Accuracy (%)**	**Precision (%CV)**
Phenytoin	46.9	51.7	110.2	5.0	49.3	105.2	3.6	48.4	103.2	4.6
	187.5	193.1	103.0	4.5	197.8	105.5	2.0	186.3	99.4	4.9
	750	777.8	103.7	3.8	777.6	103.7	4.5	751.1	100.1	1.4
4-HPPH	46.9	48.9	104.4	4.9	47.0	100.3	6.1	51.2	109.2	6.3
	187.5	180.9	96.5	4.0	186.7	99.6	5.7	190.2	101.5	1.8
	750	733.3	97.8	4.4	755.8	100.8	2.5	762.5	101.7	1.8

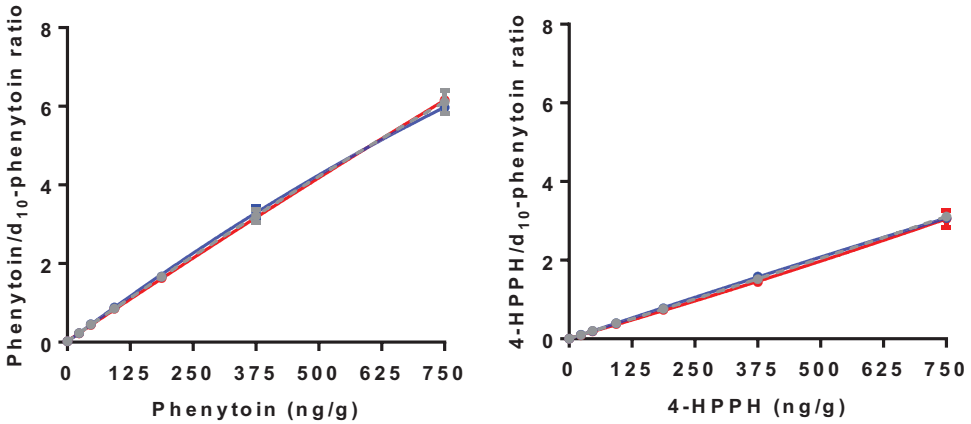

FIGURE 4 Comparison between standard curves of phenytoin (left) and 4-HPPH (right) prepared with olfactory bulbs (blue), brainstem (red), and remainder of brain tissue (grey). Variability between different regions was not significant and was within limits of the assay validated above

were prepared at three different concentrations. Equations described below were used to quantify these effects and the results are shown in Table 6.

The recovery of phenytoin and 4-HPPH from plasma and brain samples was found to be more than 98%. Both plasma and brain matrix shows ion enhance effect on

$$\%\ recovery = \frac{Peak\ area\ ratio\ of\ prespiked\ sample}{Average\ peak\ area\ ratio\ of\ postspiked\ sample(n=3)} \times 100, \quad (1)$$

$$Matrix\ effect = \left(1 - \frac{Peak\ area\ ratio\ of\ postspiked\ sample}{Average\ peak\ area\ ratio\ in\ methanol\,(n=3)}\right) \times 100. \quad (2)$$

TABLE 5 Intraday accuracy and precision of phenytoin and 4-HPPH quantification in rat brainstem and olfactory bulb homogenates based on quality control samples.

Analyte in brainstem	Nominal conc. (ng/g)	Intraday (n = 3)		
		Mean (ng/g)	Accuracy (%)	Precision (CV%)
Phenytoin	46.9	48.9	104.3	6.0
	187.5	197.8	105.5	5.8
	750	739.5	98.6	1.6
4-HPPH	46.9	50.2	107.0	4.2
	187.5	200.8	107.1	4.6
	750	744.4	99.3	4.1
Analyte in Olfactory Bulbs	**Nominal conc. (ng/mL)**	**Intraday (n = 3)**		
		Mean (ng/g)	**Accuracy (%)**	**Precision (CV%)**
Phenytoin	46.9	48.7	104.0	2.0
	187.5	187.7	100.1	3.1
	1000	758.4	101.1	0.4
4-HPPH	46.9	48.0	102.4	1.8
	187.5	187.8	100.1	2.1
	750	759.8	101.3	2.7

TABLE 6 Recovery and matrix effect on phenytoin and 4-HPPH in rat plasma and brain (n = 3).

Matrix	Concentration (ng/mL)	% Recovery		Matrix effects (%)	
		Phenytoin	4-HPPH	Phenytoin	4-HPPH
Plasma	15.6	100.9 ± 0.5	99.2 ± 1.4	−2.1 ± 1.5	−1.9 ± 1.1
	62.5	99.0 ± 0.7	97.9 ± 1.3	−2.0 ± 1.1	−1.7 ± 0.3
	250	104.2 ± 3.3	99.8 ± 0.3	−7.8 ± 2.6	−3.3 ± 0.6
Brain	46.9	101.7 ± 3.4	98.5 ± 2.5	−5.5 ± 2.6	−3.1 ± 2.2
	187.5	103.7 ± 3.1	100.5 ± 2.0	−4.3 ± 0.7	−3.5 ± 0.9
	750	110.6 ± 1.4	104.1 ± 1.5	−7.8 ± 2.3	−7.4 ± 0.8

both the analytes, phenytoin and 4-HPPH (indicated by negative values). The matrix effects were found to be within acceptable range of ±15% [37–39].

3.4 | Application of the LC-MS/MS method to the validation of a seizure model

The seizure model for which the LC-MS/MS method presented in this study was used to validate entailed rats being intravenously administered phenytoin sodium solution or saline and tested for a seizure response. Phenytoin was quantified in plasma and brain tissue of tested rats to support the attribution of an antiseizure pharmacodynamic effect observed to phenytoin, while simultaneously validating the utility of the method for in vivo investigations by showing that it could detect real sample phenytoin concentrations, consistent with those reported in the literature after a similar intravenous dose. The average plasma and brain concentrations of phenytoin from the experiment are presented in Figure 5A and B.

The average phenytoin plasma and brain concentrations were 6.96 ± 1.07 μg/mL and 7.57 ± 1.09 μg/g, respectively, however, statistically they were deemed to be indifferent ($p > 0.05$). Literature values for phenytoin plasma concentrations after i.v. administration to male rats have been reported to range from 7 to 12 μg/mL after doses of 20 to 30 mg/kg [9, 22, 40–42]. While phenytoin concentrations in the brain are less frequently measured, Ogiso et al. [41] reported a concentration of 12 μg/g after i.v. administration of a 20 mg/kg dose to male Wistar rats which provides a point of comparison to the data in the present study. Overall, the concentrations measured in our study were, therefore, similar to, albeit slightly lower than, those reported in the limited comparable studies from the literature and supported the reliability of the LC-MS/MS method and antiseizure effect seen in the seizure model. The slightly lower concentrations we observed may have been due to variations in the methodology of the studies described above (e.g., differences in dose, administration site, rat strain, rat age/weight, or analytical method sensitivity) but to some extent may also have reflected the

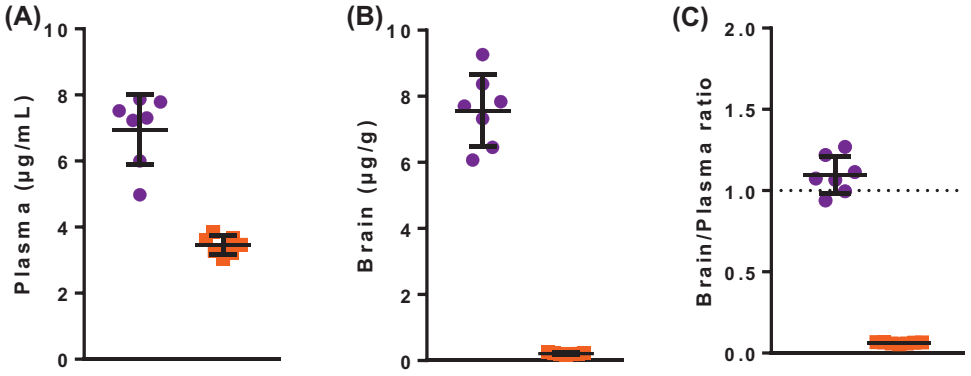

FIGURE 5 Mean plasma concentrations (A), brain concentrations (B), and brain/plasma ratio (C) of phenytoin (purple circles) and 4-HPPH (orange squares) in rats at 65 min after intravenous phenytoin administration (25 mg/kg). Error bars represent standard deviation

marginally extended time point measured in this study (65 vs. 60 min) due to the need to induce and observe a seizure at the 60-min time point, prior to euthanasia. The brain or plasma ratio of phenytoin is also presented in Figure 5C, as this represents the average ratio of phenytoin in these compartments in individual rats which is not represented in the graphs of brain and plasma concentrations of the group. The data show a trend toward a slight predominance of phenytoin in the brain compared to plasma (average ratio $= 1.10 \pm 0.12$), which is consistent with the expected distribution of the drug and the observations of Ogiso et al. [41], however, in this study it was not found to be statistically different from 1.0 ($p > 0.05$).

Concentrations of the major metabolite of phenytoin, 4-HPPH, were measured in conjunction with phenytoin in the present study to more accurately evaluate its pharmacokinetics (Figure 5A and B). The mean concentration of 4-HPPH in plasma was 3.45 ± 0.28 µg/mL, which was expected, given that 65 min had passed in which phenytoin would have begun to be metabolized. Contrary to the phenytoin results, the concentration of 4-HPPH in the brain was 0.21 ± 0.03 µg/g which was significantly lower than that found in plasma ($p < 0.05$). The marked lack of 4-HPPH in the brain compared with that in plasma was further seen in the average brain or plasma ratio of 0.06 ± 0.01, which is consistent with the increased polarity and water solubility (and therefore, poor blood–brain–barrier permeability) of the metabolite. The most comparable study to our intravenous application that was found in the literature was that of Kim et al. [22] who injected a commercial solution of phenytoin intravenously into male Sprague–Dawley rats at a dose of 25 mg/kg and found both phenytoin and 4-HPPH plasma concentrations very similar to those in the present study. No data were provided on 4-HPPH brain concentrations in this study, but the omission of brain tissue data from the figure presenting 4-HPPH tissue to plasma ratios at 30 min, while this was shown in an equivalent figure for phenytoin suggests that it was not detectable in the brain at this time point. This is consistent with the very low brain to plasma ratio of 4-HPPH shown in Figure 5C, demonstrating the poor penetration of the metabolite into the brain from plasma. The fact that some was able to be detected in the present study could be a combination of the increased sensitivity of the analytical method used here and the later time point (65 vs. 30 min) at which the sample was collected. Further support of the low brain permeability of 4-HPPH is provided by DeVane et al. [43], who were unable to detect any brain 4-HPPH, in this case in the maternal rat brain, over 16 h after a 30 mg/kg intraperitoneal dose of phenytoin. The peak 4-HPPH plasma concentration was relatively low (~1 µg/mL) in this study, possibly due to slower metabolism in female rats [44], compounded by a state of pregnancy [8].

3.5 | Application of the LC-MS/MS method to the study of intranasal drug delivery

The second application of the method was to study plasma and brain concentrations of phenytoin after intranasal administration of a phenytoin solution. Intranasal delivery of central nervous system-active drugs, such as phenytoin, is an area of growing interest in pharmaceutics, especially with regards to exploiting direct delivery pathways and bypassing the systemic barriers to successful delivery to permit lower dosages and reduced side-effects [45]. The nose is, however, limited in the volume of drug vehicle it can accommodate, so particulate systems to maximize drug delivery are currently being designed in our laboratory for testing in future studies. As an initial step toward this, the capability of the developed LC-MS/MS method to detect phenytoin and 4-HPPH concentrations after intranasal administration of a simple phenytoin solution was assessed (Figure 6).

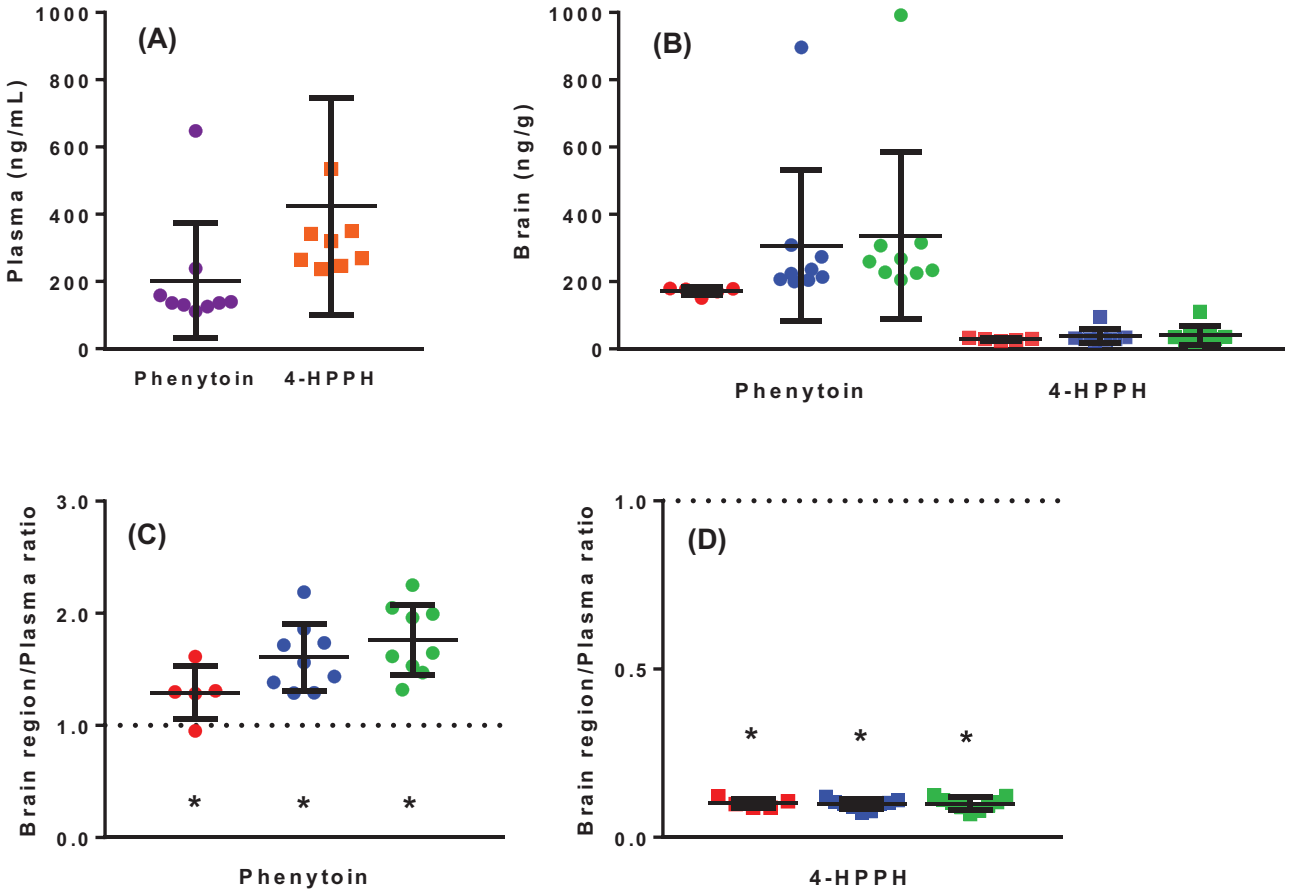

FIGURE 6 Mean phenytoin (circles) and 4-HPPH (squares) concentrations in the plasma (A) and brain (B) and brain/plasma ratios of phenytoin (C) and 4-HPPH (D) at 65 min after intranasal phenytoin solution administration. Regional brain data are presented for the olfactory bulbs (red), main brain (blue), and brainstem (green). Error bars represent standard deviation. Asterisks (*) represent ratios significantly different to 1.0 ($p < 0.05$)

The intranasal route of delivery has not previously been investigated in any detail for phenytoin, so to perform an informative experiment, the method needed to be sensitive enough to detect low concentrations of phenytoin which were likely to be encountered given the lower dose administered via this route. The method proved sensitive enough to quantify all tissue concentrations of phenytoin and 4-HPPH in this intranasal experiment (Figure 6). Mean concentration of phenytoin in the plasma was 203 ng/mL (Figure 6A) and in the brain 172, 307, and 337 ng/g in the olfactory bulbs, main brain, and brainstem, respectively (Figure 6B). No significant difference was found between plasma and the brain regions, or between the brain regions themselves ($p > 0.05$). In the case of 4-HPPH, the mean concentration in the plasma was 424 ng/mL (Figure 6A) and in the brain 29, 38, and 40 ng/g in the respective regions as listed above (Figure 6B). No significant difference was found between the brain regions, however, as with the intravenous experiment, the concentration in plasma was significantly higher than any brain region ($p < 0.05$). As stated earlier, the LLOQ for phenytoin and 4-HPPH in this study was 7.81 ng/mL for plasma and 23.4 ng/g for brain tissue showing that the assay has ample range to detect lower concentrations in future studies of other phenytoin intranasal delivery systems, if necessary. For comparison, the sensitivity of previously reported methods for phenytoin (and occasionally 4-HPPH) measurement in rat plasma or brain have been restricted to lower limits of quantification between 50 and 500 ng/mL, as these studies have generally dealt with typical systemic doses of phenytoin which produce high plasma and brain concentrations [5, 9, 23–25, 46].

The ability to measure brain concentrations in different brain regions in this method offered some insight into potential direct intranasal routes of delivery. A number of direct delivery pathways have been proposed, most notably those passing through or peripheral to the olfactory or trigeminal neurons which extend from the CNS and innervate the nasal epithelium via the olfactory bulbs or brainstem, respectively [45, 47] hence, the rationale for analyzing these regions. The most important observation in this respect was that the olfactory bulb to plasma ratio

of phenytoin was significantly lower than the brainstem to plasma ratio ($p < 0.05$) (Figure 6C). While the difference between the olfactory bulb and brainstem concentrations was not statistically significant between the actual concentrations (Figure 6B), this was clearly due to the presence of the outlier in the upper range which, when omitted from analysis, revealed the same significant difference ($p < 0.05$) that the ratio to plasma data had corrected for.

This observation is interesting as it is in contrast to studies by Czapp et al. [48] and Serralheiro et al. [49, 50] who have previously reported higher concentrations of their respective model antiseizure drugs phenobarbital, lamotrigine, and carbamazepine in the olfactory bulbs relative to the brain after intranasal delivery. While they suggested direct delivery of antiseizure drugs via an olfactory neuron pathway, our data suggest a lack of accumulation of phenytoin in the olfactory bulbs. It should be noted that they all used hydrogel delivery systems which may have changed the delivery pathway by increasing mucoadhesion and retention on the nasal epithelium in contrast to the free solution tested in the present study. Czapp et al. [48] also tested a free drug solution and found overall lower brain delivery than after the hydrogel, but the relative proportion in the olfactory bulbs was not presented so was unable to be used to provide any additional comparison. Other factors that may have influenced the drug delivery profile include the molecular properties of the drugs themselves, such as relative lipophilicity and intranasal delivery techniques. With respect to the latter, the animals in the studies mentioned above were all anesthetized and kept supine for much longer periods than in the present study in which rats quickly regained consciousness and were moving around freely for most of the experiment, removing biases which can increase nasal absorption including impairment of mucociliary clearance, drainage, and mechanical removal (i.e., sneezing and snorting) [51].

Finally, it is worth discussing the measurement of 4-HPPH in the intranasal experiment, which was included to gain added insight into the movement of phenytoin in this situation of uncertain pharmacokinetic behaviour. The first obvious difference to the intravenous experiment discussed earlier was that the 4-HPPH concentration in plasma was higher than phenytoin (Figure 6A). This was to be expected given the lower dose that was administered in the intranasal experiment. In the seizure model validation, the plasma concentrations fell within the saturable range of phenytoin metabolism, so phenytoin concentrations would have increased above 4-HPPH when the enzyme capacity was reached. In the intranasal experiment, however, the metabolic capacity appears to have been sufficient to deal with the amount of phenytoin in the plasma. In addition, it is possible that some phenytoin metabolism may have occurred in the nasal passage, which is known to contain CYP450 enzymes and may have contributed to the plasma concentrations of 4-HPPH [52]. On this line of thought, it is also notable that the concentration of 4-HPPH in the brain regions relative to plasma was significantly higher, as seen in the brain to plasma ratios (0.1 in all regions compared with 0.06 in the intravenous experiment) ($p < 0.05$) (Figure 6B and D). This was unexpected as the plasma concentrations of 4-HPPH were much higher in the intravenous experiment which would have been expected to create a greater concentration gradient and result in more 4-HPPH crossing the blood–brain barrier, despite its poor permeability as discussed earlier. A possible explanation for this could be some metabolism of phenytoin in the nasal epithelium followed by a delivery of 4-HPPH to the brain via direct intranasal pathways and offers additional evidence for the existence and potential exploitation of such routes. Using the method outlined in the present study to measure 4-HPPH in future studies investigating directly comparable systemic dosing and other intranasal phenytoin delivery vehicles as discussed above will provide material for extended discussion of these findings.

4 | CONCLUSIONS

Phenytoin is a highly effective antiseizure drug, but it suffers from poor water solubility and pharmacokinetic issues including saturable metabolism to its major metabolite, 4-HPPH, and efflux by P-gp. This makes it a very interesting model drug for pharmaceutical delivery studies, however, validated and sensitive analytical methods to study it in rat tissues are lacking. In this manuscript, we have presented the development and validation of a simple and sensitive LC-MS/MS method for the determination of phenytoin and its major metabolite, 4-HPPH, in the plasma and brains of rats. The application of the method has been demonstrated through two in vivo investigations; the validation of a seizure model with intravenous phenytoin and the investigation of intranasal phenytoin delivery. The first served as an additional validation of method accuracy by quantifying concentrations of phenytoin and 4-HPPH which were consistent with other studies of intravenous phenytoin, while the second served as a pioneering investigation into delivery of phenytoin to the brain by the potentially advantageous intranasal route. To our knowledge, our data present the most sensitive validated analytical method for phenytoin and 4-HPPH quantification in rat plasma and brain in the literature and the only method which utilizes LC-MS/MS rather than HPLC for these tissues. We anticipate it to be very useful for any future investigations, employing phenytoin

as a model drug, particularly in the context of more complex intranasal delivery systems such as those under development in our own laboratory.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

DATA AVAILABILITY STATEMENT

Research data are not shared.

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