ORIGINAL ARTICLE

Nonlinear protein binding of phenytoin in clinical practice: Development and validation of a mechanistic prediction model

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Aims: To individualize treatment, phenytoin doses are adjusted based on free concentrations, either measured or calculated from total concentrations. As a mechanistic protein binding model may more accurately reflect the protein binding of phenytoin than the empirical Winter–Tozer equation that is routinely used for calculation of free concentrations, we aimed to develop and validate a mechanistic phenytoin protein binding model.

Methods: Data were extracted from routine clinical practice. A mechanistic drug protein binding model was developed using nonlinear mixed effects modelling in a development dataset. The predictive performance of the mechanistic model was then compared with the performance of the Winter–Tozer equation in 5 external datasets.

Results: We found that in the clinically relevant concentration range, phenytoin protein binding is not only affected by serum albumin concentrations and presence of severe renal dysfunction, but is also concentration dependent. Furthermore, the developed mechanistic model outperformed the Winter–Tozer equation in 4 out of 5 datasets in predicting free concentrations in various populations.

Conclusions: Clinicians should be aware that the free fraction changes when phenytoin exposure changes. A mechanistic binding model may facilitate prediction of free phenytoin concentrations from total concentrations, for example for dose individualization in the clinic.

KEYWORDS

fraction unbound, free, in vivo, phenytoin, protein binding

1 | INTRODUCTION

Phenytoin is an effective drug for treatment of generalized and focal seizures. It putatively acts by causing voltage-dependent block of voltage gated sodium channels.[1] Phenytoin is, however, considered a drug with a small therapeutic window: low exposure may result in inadequate therapy and high exposure may even lead to serious neurological side effects. Careful dose titrating is, thus, warranted to prevent

The authors confirm that the PI for this paper is Rob ter Heine.

inadequate drug exposure. There is, therefore, general consensus that dosing of phenytoin should be individualized based on therapeutic drug monitoring (TDM).[2] For purposes of TDM, a therapeutic range of 10–20 mg/L is usually advocated for the total plasma concentrations and a range of 1–2 mg/L for the free plasma concentrations, assuming a 10% free fraction of phenytoin on the population level.[2,3] Dose individualization of phenytoin is hampered by its nonlinear pharmacokinetics, its variable protein binding and drug–drug interactions. In the therapeutic range, the elimination pathway of phenytoin can become saturated and an increase in dose may result in more than dose-proportional

increases of plasma concentrations.[4] Furthermore, phenytoin is highly, yet variably, bound to serum albumin.[5,6] As the free phenytoin concentration is responsible for the pharmacological effect, measurement of free concentrations for TDM purposes is preferred over measurement of total serum concentrations.[2] However, in routine clinical practice, often only total concentrations are measured, since this is associated with less cost, a faster time to results and simpler analytical procedures compared to the quantification of free concentrations. As variation in serum albumin concentrations is the main driver for variable protein binding of phenytoin, total concentrations are often corrected for hypoalbuminaemia to interpret measured concentrations in absence of a measured free concentration. The Winter–Tozer formula has been the gold standard for this purpose in recent decades.[7] With this formula, the free phenytoin concentration may be approximated, based on the total concentration, serum albumin concentration and knowledge on the presence of severe renal dysfunction (SRD), as uraemic toxins may displace phenytoin from albumin.[8]

The predictive performance of the Winter–Tozer equation has often been challenged as factors other than albumin or severe renal dysfunction are known to impact the free fraction of phenytoin.[9] For example, concomitant valproic acid use and temperature of sample handling are known to impact the free fraction of phenytoin,[10] as valproic acid may displace phenytoin from albumin and the phenytoin–albumin dissociation constant is temperature dependent.[11] Furthermore, the empirical Winter–Tozer equation assumes a concentration-independent free fraction and, thereby, potentially ignores nonlinear binding kinetics.[12] Although several groups have investigated the free phenytoin pharmacokinetics in population pharmacokinetic models,[13,14] as it stands, no mechanistical model has thus far been developed for the in-vivo phenytoin binding kinetics of phenytoin. As, from a theoretical standpoint, a mechanistic protein binding model may more accurately reflect the protein binding of phenytoin than an empirical model, e.g. the Winter–Tozer equation, our aims were to: (i) develop a mechanistic protein binding model for phenytoin; and (ii) to compare the predictive performance of the mechanistic model with the Winter–Tozer equation. Translating the findings of such a mechanistic model may facilitate improved predictions of free phenytoin concentrations from total concentration measurements for purposes of dose individualization in the clinic.

2 | METHODS

2.1 | General approach

We collected development and validation datasets with phenytoin protein binding data from routine clinical practice. The development dataset was used to develop a mechanistic model for phenytoin protein binding. External evaluation was then performed with the validation dataset from the same clinic and 4 other datasets. For this purpose, the predictive performance of the mechanistic model was compared with that of the Winter–Tozer equation.

What is already known about this subject

- Phenytoin is a pharmacotherapeutic cornerstone for treatment of epilepsy. Dosing individualization of phenytoin is hampered by nonlinear pharmacokinetics and variable protein binding.

What this study adds

- We show that protein binding of phenytoin is concentration-dependent and that the traditionally used Winter–Tozer equation to predict free concentrations is inferior compared to a mechanistic protein binding model.

2.2 | Data collection

We collected 1 model development dataset and 5 validation sets (validation dataset 1–5) from routine clinical practice, containing paired observations of total and free phenytoin concentrations and information on the relevant covariates of serum albumin concentration, severe renal dysfunction and concomitant valproic acid use.

2.2.1 | Development dataset and validation dataset 1

For the model development dataset and validation dataset 1, we collected data from the Utrecht Patient Oriented Database (UPOD).[15] Besides paired observations of total and free phenytoin concentrations, data on serum albumin concentrations and blood urea nitrogen were collected within 48 h before and after the phenytoin concentration observations. Furthermore, information on age and sex were collected as well as the concomitant use of valproic acid, based on its recorded use. Presence of severe renal dysfunction was based on a blood urea nitrogen concentration of 14 mmol/L (39.2 mg/dL) or higher[16] and coded as a binary covariate. Phenytoin concentrations were measured using a validated fluorescent polarization immunoassay (FPIA) or liquid chromatography coupled with tandem mass spectrometry assay. Free phenytoin concentrations were obtained by ultracentrifugation at ambient temperature. For the development dataset, data were collected from UPOD between January 2005 and January 2011. For validation dataset 1, data were collected from the UPOD database from January 2011 until November 2017. The institutional review board approved the use of these anonymized data for our research. A pragmatic approach was used for splitting of the dataset for the development dataset and validation set 1. The initial extraction was performed in 2011 and based on these data a mechanistic model was developed. Since new data had been collected in routine practice since this last extraction, a validation dataset was collected in the time frame after 2011.

2.2.2 | Validation datasets 2–5

For external validation sets, we searched PubMed for recent research with the following keywords: "phenytoin", "free", "fraction", "sheiner-

tozer", "winter-tozer". The retrieved publications were screened for relevant literature. The corresponding authors were contacted with a request for data sharing. Selection of these data were based on willingness of the researchers to share their data and the presence of information on total and free phenytoin concentrations, as well as data on renal function and concomitant valproic acid use.

2.3 | Data analysis

2.3.1 | Model development

Statistical analysis of the data was performed by means of nonlinear mixed effects modelling with the software package NONMEM V7.4.1 using the prediction ($PRED) subroutine. Throughout the analysis, the first order conditional estimation method was used. Parameter uncertainty was calculated with the covariance option in NONMEM and presented as relative standard error of the estimate (RSE).

We developed a protein binding model, independent of the pharmacokinetics of phenytoin. Therefore, in our model, the free concentration (C_{free}) was used as dependent variable. The dependent variable was predicted from the total concentration and serum albumin concentrations under the assumption of 1 site specific binding, as described by equation 1.[12]

Equation 1: 1 site specific binding

$$C_{total} = C_{free} + \frac{B_{max} \times C_{free}}{K_D + C_{free}}$$

In this equation, C_{total} is the total concentration (mg/L), C_{free} is the free concentration (mg/L), B_{max} is the maximum binding constant (mg/L) and K_D is the dissociation constant (mg/L). With the assumption that the free fraction (F_U) can be described with equation 2,[12] equation 1 and 2 can be arranged to equation 3. It follows from equation 2 that if the free concentration is negligible when compared to K_D, the free fraction is independent of the free concentration and that if the observed free concentrations are of the approximate same order of magnitude as the K_D, there will be a positive correlation between the free concentration and the free fraction.[12]

Equation 2: Free fraction

$$F_U = \frac{C_{free}}{C_{total}} = \frac{K_D + C_{free}}{B_{max} + K_D + C_{free}}$$

Equation 3: Free fraction for 1 site specific binding model

$$F_U = \frac{-K_D - B_{max} + C_{total} + \sqrt{(K_D + B_{max} - C_{total})^2 + 4^* C_{total}^* K_D}}{2^* C_{total}}$$

In the base model, the dependent variable C_{free} was calculated from the free fraction from equation 3 and the measured total concentration. In the base model, the maximum binding capacity was assumed to be proportionally related to serum albumin concentrations and this relationship was calculated as described in equation 4.

Equation 4: relationship between serum albumin and B_{max}

$$B_{max} = \Theta_{ALB1} \times (1 + (\Theta_{ALB2} \times (ALB_i - 27)))$$

In this equation, Θ_{ALB1} is the estimate for B_{max} for an individual with a serum albumin of 27 g/L, Θ_{ALB2} is the estimate for the gradient of change in B_{max} when the individual serum albumin concentration (ALB_i, in g/L) differs from 27 g/L. After fitting of a specific binding model with 1 site, we tested the presence of a second binding site with both specific binding as nonspecific binding.

Interindividual variability in K_D, that may originate from interindividual differences in affinity of the interaction between albumin and phenytoin, was assumed to be log-normally distributed and for the residual error we tested additive, proportional and combined additive and proportional error models.

After fitting of the base model, we tested concomitant valproic acid use and severe renal dysfunction as binary covariates for B_{max} using a forward inclusion backward elimination approach. These covariates were tested on B_{max}, because drug protein displacement causes are represented by B_{max} and not by a change in individual affinity between the drug and the protein, which is described by K_D. The relative change in B_{max} was calculated by multiplying the B_{max} (equation 4) with Θ_{SRD}^{COV} or Θ_{VALP}^{COV}, where COV has the value of 1 or 0 in, respectively, the presence or absence of the binary covariate. The parameters Θ_{SRD} and Θ_{VALP} can then be interpreted in the fractional change of B_{max} as a result of the presence of severe renal dysfunction and concomitant valproic acid use, respectively. A binary covariate for severe renal dysfunction was chosen as it is known that protein binding is only relevantly affected in severe renal dysfunction.

Statistical significance during likelihood ratio testing of nested models was derived from the objective function in NONMEM, that follows a χ^2 distribution. A level of significance of $P < .01$ was used for both the forward inclusion and the backward elimination.

2.3.2 | Validation

For validation purposes, we compared the performance of the developed mechanistic model and the Winter–Tozer equation (equation 5) to predict the free concentration in the validation datasets. Predictive performance was assessed with the mean prediction error (MPE) and root mean squared error (RMSE), as proposed by Sheiner et al.[17] For calculation of the predictive performance of the mechanistic model, we calculated the unbound concentration from the total concentrations and covariates from using the population parameter estimates from our model.

Equation 5

$$C_{free} = 0.1 \times \frac{C_{total}}{a \times ALB_i + 0.1}$$
$$a = 0.2 \text{ in normal renal function}$$
$$a = 0.1 \text{ in serve renal dysfunction}$$

In this equation, ALB_i is the individual serum albumin concentration in g/dL.

TABLE 1 Summary of datasets

	Development dataset	Validation dataset 1	Validation dataset 2 Kane et al.	Validation dataset 3 Soriano et al.	Validation dataset 4 Joerger et al.	Validation dataset 5 Krasowski and Penrod
Number of paired observations of total unbound concentrations	379	115	140	21	266	1753
Number of individuals	144	53	140	21	151	756
Age (y), median (range)	34 (0–92)	40 (0–77)	54 (20–83)	57 (21–86)	50.5 (10–85)	55.7 (0–96)
Male/female (%/%)	56%/44%	53%/47%	49%/51%	48%/52%	47%/53%	56%/44%
Number of cases with concomitant valproic acid use (percentage of total)	36 (9.5%)	13 (11.3%)	0 (0%)	0 (0%)	42 (15.8%)	177 (10%)
Number of cases with severe renal dysfunction (percentage of total)	26 (6.9%)	9 (7.8%)	4 (2.9%)	21 (100%)	0 (0%)	118 (6.7%)
Population selection	Cross-sectional	Cross-sectional	Neuro-intensive care patients	End-stage renal disease patients	Cancer patients	Cross-sectional
Serum albumin median (range)	27.6 g/L (10–47.5)	28.9 g/L (11.0–51.9)	34 g/L (14–47)	30 g/L (16–42)	40 g/L (13–55)	30 g/L (10–60)
Bioanalytical method[a]	FPIA or LCMS[b]	LCMS	FPIA	FPIA	FPIA	EMIT
Collection of unbound fraction	Ultracentrifugation at ambient temperature	Ultracentrifugation at ambient temperature	Ultracentrifugation at ambient temperature	Ultracentrifugation at ambient temperature	Ultracentrifugation at ambient temperature	Ultracentrifugation at ambient temperature

Ranges in this table are minimal–maximal ranges of the observed values.

[a]EMIT: enzyme-multiplied immunoassay technique; FPIA: fluorescence polarization immunoassay, LCMS: liquid chromatography coupled with tandem mass spectrometry.

[b]Both methods were cross validated according to in-house methods.

2.4 | Nomenclature of targets and ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY,[18] and are permanently archived in the Concise Guide to PHARMACOLOGY 2017/18.[19]

3 | RESULTS

3.1 | Data extraction

Results of the data extraction are shown in Table 1, together with the characteristics of the external datasets. In the development dataset we had a total of 379 paired observations of total and free phenytoin concentrations in 144 individuals with age ranging from 0 to 92 years. In validation dataset 1, a total of 115 paired observations in 53 individuals were collected.

The validation datasets 2–5 were respectively obtained from previous research by Kane et al.,[20] Soriano et al.,[21] Joerger et al.,[22] and Krasowski and Penrod.[23] The studied populations in these validation sets were either selected cross-sectionally, or from end-stage renal disease patients, neuro-intensive care patients or cancer patients. For the details of the data collection process and population types we would like to refer to the original manuscripts. In validation dataset 2, by Kane et al.,[20] severe renal dysfunction was coded as blood urea nitrogen >14 mmol/L. The validation dataset 4, by Joerger et al.,[22] contained some cases with missing covariate data, and these were excluded from the dataset. A summary of all datasets can be found in Table 1. Validation dataset 5, by Krasowski and Penrod,[23] contained data from patients that had paired total and free phenytoin measurements along with a serum/plasma albumin measurement within 7 days of the phenytoin values. The original dataset was updated to identify patients with severe renal dysfunction with blood urea nitrogen concentration of 14 mmol/L or higher (approved as retrospective study by University of Iowa Institutional Review Board, protocol # 201610760).

3.1.1 | Exploratory analysis of the development dataset

As expected, the observed free fraction increased at lower serum albumin concentrations. A significant negative correlation was found with a Spearman's ρ of −0.678 (P << .001). In the development dataset concentrations up to approximately 9 mg/L were observed, showing that in routine clinical practice supratherapeutic concentrations are not uncommon. Surprisingly, we observed a highly significant positive correlation between free phenytoin concentrations and the free fraction (Spearman's ρ 0.414, P << .001), indicating that the free fraction depends on the free concentration and that, therefore, the observed free concentrations are in the same order of magnitude as the K_D. Although the relationship was highly significant, the correlation was moderate (Spearman's ρ 0.414), which led to variability in the free fraction, potentially caused by protein displacement of phenytoin by other drugs or uraemic toxins, variable albumin concentrations and bioanalytical assay error. Figure 1 shows the plotted free fraction *vs* albumin and free concentration in panel A and B, respectively.

3.1.2 | Model development

We successfully fit the proposed mechanistic binding model to the observed data. A proportional error model best described the residual error. A second specific or nonspecific binding site could not be identified in our data. The binary covariates severe renal dysfunction and concomitant valproic acid use significantly decreased the maximum binding capacity of albumin for phenytoin when tested univariate (P < .01). When including both parameters in the model, the model fit was even further improved (P < .0001), showing that severe renal dysfunction and concomitant valproic acid use both and independently explain variability in B_{max}. Both covariates were, therefore, retained in the final model. In the final model, severe renal dysfunction and concomitant valproic acid use decreased the B_{max}, respectively, by 14.7% (Θ_{SRD} = 0.853, RSE 5%) and 30.2% (Θ_{VALP} = 0.698, RSE 4%), respectively. These parameters could be accurately assessed in the
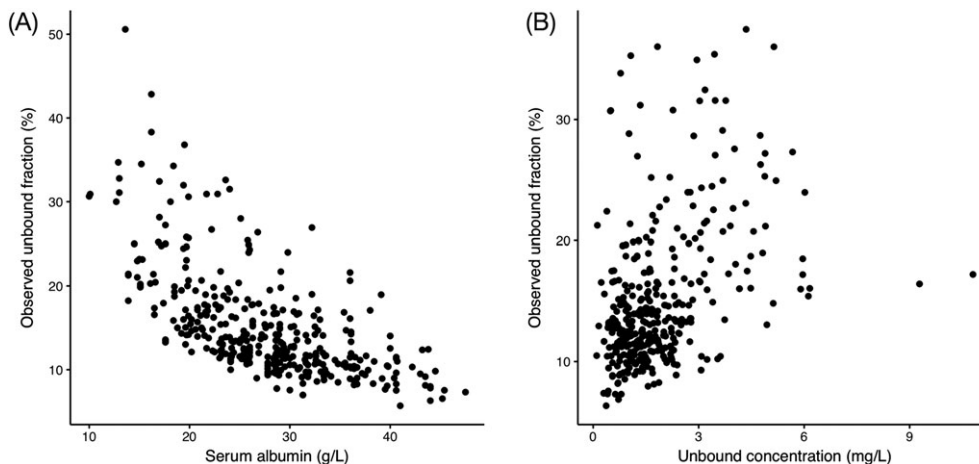

FIGURE 1 Free fraction *vs* serum albumin (A) free phenytoin (B) concentrations in the development dataset

TABLE 2 Parameter estimates for the base and final model

Parameter	Base model Estimate (RSE)	Final model Estimate (RSE)
Θ_{ALB1} (mg/L)	105 (16%)	117 (14%)
Θ_{ALB2} (L/g)	0.0325 (6%)	0.0345 (5%)
Θ_{SRD}	-	0.0853 (5%)
Θ_{VALP}	-	0.698 (4%)
K_D (mg/L)	15.1 (18%)	16.2 (15%)
Inter individual variability K_D (%)	26.6% (8%)	24.8% (8%)
Residual variability (%)	14.8% (9%)	13.2% (9%)
Objective function	−521	−600

development dataset, supported by the low relative standard error of the estimates.

The parameter estimates and for the base and final model can be found in Table 2. Goodness-of-fit plots of the final model are shown in Figure 2. As can be seen in panel A and B of this figure, the observed *vs* the individual and population predicted concentrations (black dots) are evenly distributed around the line of unity (solid black line). Furthermore, the dashed trend line with confidence interval (grey area) closely resembles the line of unity, indicating a good model fit. As observed in panel C, the normalized prediction distribution errors are evenly scattered around zero and do not show a trend when plotted *vs* the population predicted free concentration, indicating an unbiased prediction across the complete concentration range. Lastly, it can be observed in panel D that the simulated and observed data are well-
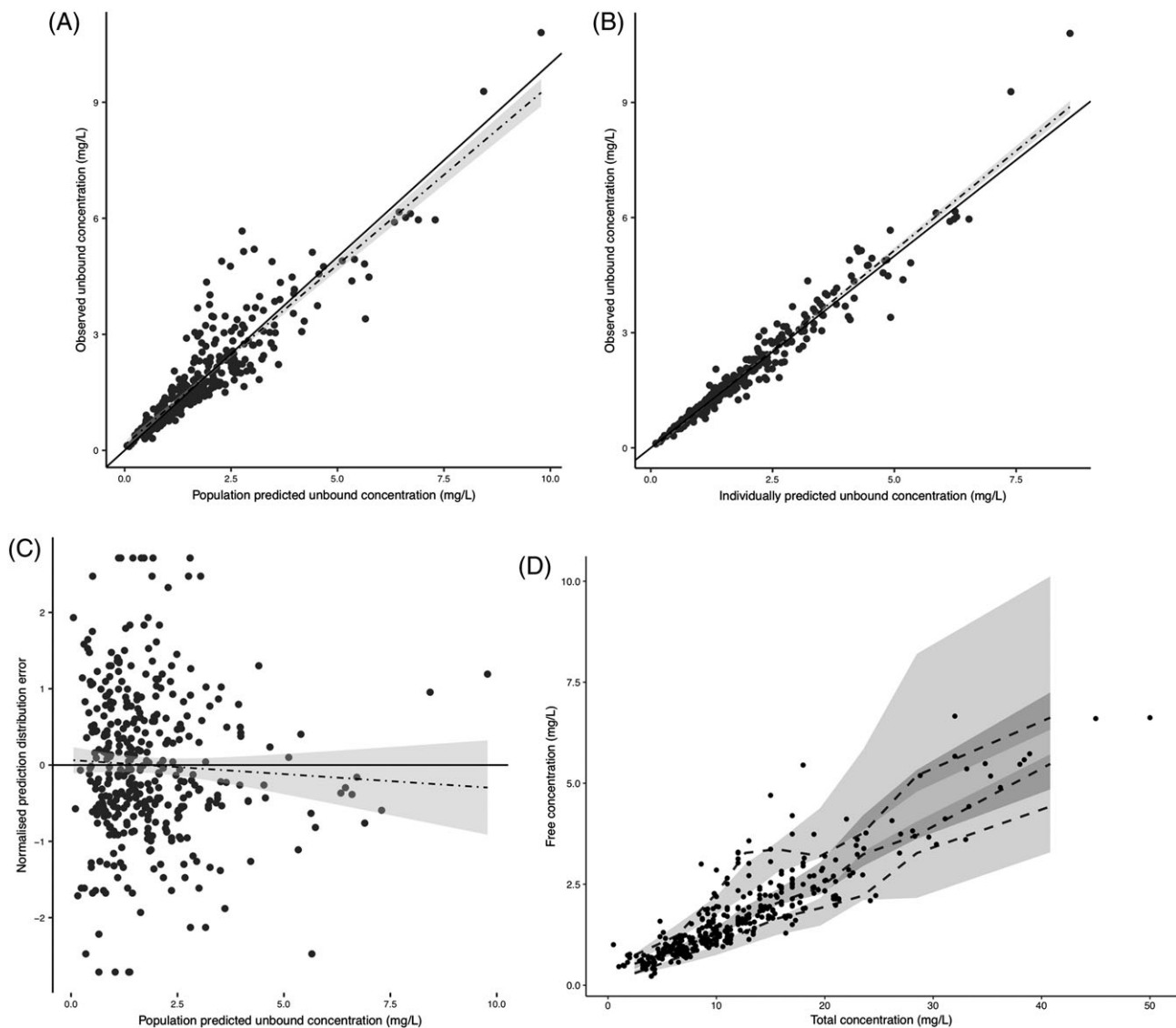
FIGURE 2 Goodness-of-fit plots. (A) observed *vs* population predicted free phenytoin concentrations. (B) observed *vs* individually predicted free phenytoin concentrations. (C) normalized prediction distribution error *vs* population predicted free phenytoin concentration. (D) prediction-corrected visual predictive check of the free *vs* the total concentration, based on 1000 simulations

TABLE 3 Validation results

| | Validation dataset 1 | | Validation dataset 2 Kane et al. | | Validation dataset 3 Soriano et al. | | Validation dataset 4 Joerger et al. | | Validation dataset 5 Krasowski et al. | |
	Mechanistic	Winter–Tozer	Mechanistic	Winter–Tozer	Mechanistic	Winter–Tozer	Mechanistic	Winter–Tozer	Mechanistic	Winter–Tozer
MPE	−2.2%	−14.8	−20.2%	−40.5%	1.1%	−74.6%	1.1%	−13.6%	17.8%	6.0%
RMSE	0.45 mg/L	0.50 mg/L	0.48 mg/L	0.66 mg/L	1.00 mg/L	1.76 mg/L	0.48 mg/L	0.41 mg/L	0.745 mg/L	0.688 mg/L

MPE, mean prediction error; RMSE, root mean squared error

aligned in the prediction-corrected visual predictive check. In this panel, the scattered dots are the observed prediction-corrected data and the dashed lines the observed 10th, 50th and 90th percentiles. The shaded areas are the prediction intervals for these respective percentiles.

3.1.3 | Validation

After the development of the mechanistic binding model in the development dataset, we compared the predictive performance of the mechanistic model and the Winter–Tozer equation (equation 5) on independent datasets. The results are presented in Table 3. In this table, the predictive performance of the developed mechanistic model and the Winter–Tozer equation are presented side by side per dataset.

As observed in Table 3, the mechanistic model consequently resulted in superior accuracy in validation set 1–4, as observed in the reduced bias (mean prediction error) of the mechanistic model when compared to the Winter–Tozer equation. Worse performance in terms of accuracy was observed for validation dataset 5. In terms of precision, the mechanistic model performed better for validation sets 1–3, as observed in the lower RMSE.

4 | CONCLUSION AND DISCUSSION

To the best of our knowledge, we are the first to report and characterize the nonlinear *in vivo* protein binding of phenytoin. Our findings indicate that the empirical Winter–Tozer equation, which assumes a fixed free fraction in the clinically relevant concentration range. The observed free phenytoin concentrations in our dataset were in the same order of magnitude as the K_D and allowed assessment of the nonlinear protein binding. The clinical implications of these findings are that, besides saturable clearance of phenytoin, saturable protein binding complicates dose titrations. When a dose is increased or reduced, while the serum albumin concentrations are stable, one should consider the possibility of an altered free fraction. Second, we showed that, in general, the mechanistic model had superior predictive performance over the Winter–Tozer equation to predict free phenytoin in external validation datasets, in terms of bias (in 4 out of 5 validation datasets) and precision (in 3 out of 5 validation datasets).

Third, despite the superior predictive performance of the mechanistic model in the majority of the external validation datasets, the predictive precision (RMSE) varied from 0.45 to 1.00 mg/L for the mechanistic model. Considering the fact that the precision (RMSE) of our model was in the same order of magnitude as the therapeutic range of free phenytoin (1–2 mg/L), we conclude that even a mechanistic model for phenytoin protein binding should not be considered an alternative for measuring free concentrations for purposes of TDM. Nonetheless, if this is not possible, a mechanistic model to predict the free concentration should be preferred over the Winter–Tozer equation. Furthermore, it appeared that validation dataset 5 was distinct from the other datasets, as the mechanistic model consequently

showed poor predictive performance in comparison with Winter–Tozer equation.

The free phenytoin measurements in validation dataset 5 used ultrafiltration methods similar to datasets 1–4 but were obtained by enzyme-multiplied immunoassay technique (EMIT) using the Syva EMIT 2000 assay. The other datasets used FPIA or LC/MS as the method for free phenytoin measurement. There is little published literature comparing free phenytoin measurements using immunoassays from different manufacturers, but a study by Roberts et al. in 2001 compared 4 different immunoassays for free phenytoin determinations.[24] This study included comparisons of EMIT and FPIA methods, showing differences in bias between methods relative to high-performance liquid chromatography as the gold standard. To our knowledge, there are no publications since the 2001 Roberts et al. study comparing free phenytoin measurements; thus, it is unknown if these differences between EMIT and FPIA methods occur with more recent instrument platforms and immunoassay versions and this should be subject for further study. Nonetheless, our analysis shows the necessity of external validation of the current model in a new population and assay method before it is applied in routine clinical practice.

One may argue that the use of blood urea nitrogen as a covariate in our study may have limitations, as other factors other than renal function, such as hydration state or high protein diet, may impact blood urea nitrogen levels. Nonetheless, as the hypothesis is that uraemic toxins are responsible for the displacement of phenytoin from albumin, we think that blood urea nitrogen is an adequate surrogate marker for uraemia.[25] In our data, we could identify interindividual variability in the binding constant K_D, as a source for variability in the free fraction. Although knowledge on this variability is not necessarily useful to predict the free phenytoin concentration from a total concentration, the mechanistic model including all sources of variability may be implemented in an existing or new population-pharmacokinetic model of phenytoin to predict individual free concentrations, e.g. to perform TDM. Previous observations of the free concentrations in an individual may then be used to more accurately predict a future free concentration. A methodological limitation of our approach may nonetheless be that in our model we assume that there is no error in the independent variable. However, since it is likely that the error in the independent variable (total concentration) is equal to or less than that of the error in the dependent variable (free concentration) and because our analysis reflects the situation that will be used in clinical practice, we think our approach is justified.

Our *in vivo* protein binding data of phenytoin were all retrospectively collected from routine clinical care. Although a real-world setting like this has many advantages, as it truly resembles clinical practice, one should realize that this approach may have limitations. For example, cases with incomplete covariate data were discarded from the development dataset. In clinical practice, laboratory measurements are performed as a part of a diagnosis or in the context of clinical disease monitoring. Including only individuals with complete data on renal function and serum albumin, may therefore introduce a selection bias. Consequently, the generalizability of our findings to other populations should be studied. Nonetheless, the saturable protein binding of phenytoin in the clinically relevant concentration range is a drug-specific characteristic and not a population-specific characteristic. Therefore, this phenomenon should be considered in other populations as well. Furthermore, the retrospective nature of our data collection strategy may have introduced bias. For example, if no data on concomitant valproic acid use were found, we concluded that the individual did not use valproic acid. Although we consider our database to be exhaustive, missing data may not be ruled out. In the latter example, this may have resulted in under-estimation of the effect of concomitant valproic acid use on the protein binding of phenytoin. Although valproic acid is most notorious for displacement of phenytoin from albumin, other less frequently used drugs such as salicylic acid and some sulfonamides have been reported to be able to displace phenytoin from albumin.[26] These drugs have not been investigated as covariates for protein binding in our model nor have they been incorporated in the Winter–Tozer equation, which is considered the gold standard for protein binding correction in the clinic. Despite these limitations, our main finding, that the phenytoin protein binding is saturable in clinically representative concentrations, still holds. Importantly, our mechanistic model outperformed the empirical Winter–Tozer equation in the majority of the external validation datasets of various populations, indicating that our model performs relatively well in different settings. Finally, future studies are recommended to validate the equations' impact on actual patient efficacy and toxicity outcomes and to quantify the effect of other covariates on protein binding of phenytoin, for example other drugs and the bioanalytical assay type.

COMPETING INTERESTS

There are no competing interests to declare.

CONTRIBUTORS

R.t.H. designed the study, performed the analysis and wrote the manuscript. S.P.K. collected data and wrote the manuscript. A.D.R.H. collected data and wrote the manuscript. M.D.K. collected data and wrote the manuscript. E.M.v.M. collected data and wrote the manuscript.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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