

**Contributions to an improved
Phenytoin monitoring and dosing
in hospitalized patients**

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Dedicated to Christian and Manon Aimée

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1 ABBREVIATIONS

aCSF	artificial cerebrospinal fluid
ADR	adverse drug reaction
BF	Bayesian Forecasting
CD	conventional dosing
dphCF	calculated free phenytoin
dphF	measured free phenytoin concentration in serum
dphT	total phenytoin in serum
FDA	Food and Drug Administration
fF	free fraction (of a drug)
GC	gas chromatography
GC-MS	gas chromatography – mass spectrometry
ICU	intensive care unit
IS	internal standard
ISO	International Organization for Standardization
IV	intravenous
LOD	limit of detection
LOQ	limit of quantification
MPPH	5-(p-methylphenyl)-5-phenylhydantoin (IS); C ₁₆ H ₁₄ N ₂ O ₂
MS	mass spectrometry
PD	Pharmakodynamics
PHT	Phenytoin (5, 5-Diphenylhydantoin); C ₁₅ H ₁₂ N ₂ O ₂
PK	Pharmacokinetics
QC	quality control
RSD	relative standard deviation
RT	retention time
SD	standard deviation
SPC	summary of product characteristics
SPE	solid phase extraction
SST	system suitability test
TDM	Therapeutic Drug Monitoring
TSMH	trimethylsulfonium hydroxide

2 INTRODUCTION

The importance of Phenytoin in the prevention and therapy of epilepsy

Epilepsy is the most common serious neurological condition affecting an estimated 50 million people (1% of the world population) worldwide [1-2]. The annual incidence ranges from 20 to 70 cases per 100'000, and the prevalence is 0.4 to 0.8% [1].

The major goal in epilepsy is to stop seizures or to minimize their frequency and also to have minimal concurrent undesirable side-effects [2].

Generalized epilepsies occur in approximately one-third of patients [1]. Phenytoin (PHT) is one of the most efficacious, oldest, and widely prescribed anticonvulsants for the treatment of epilepsy [3]. PHT influences the voltage-activated sodium, potassium and calcium channels [1].

Different studies showed that antiepileptic prophylaxis with PHT in adults with severe traumatic brain injuries before and after neurosurgical intervention is effective, whereas the risk for an early (until seven days after the neurosurgical intervention) posttraumatic seizure after acute, traumatic brain injuries can be diminished significantly [4-7].

The use of antiepileptic drugs to treat patients who have developed post-traumatic epilepsy is standard [8]. Prophylactic treatment with PHT, beginning with an IV loading dose, should be initiated as soon as possible after injury to decrease the risk of post-traumatic seizures occurring within the first seven days [8]. PHT is often used as antiepileptic (study) drug because it is available as a parenteral formulation (rapid loading dose), and its use in the management of acute seizures is widely established with respect to both efficacy and safety and the drug is authorized in this indication. [9-10]. PHT is the only antiepileptic for which an optimal range of serum concentrations (therapeutic range) has been defined clearly and is effective in preventing early seizures after acute brain injury [4, 11].

Chemical and physical characteristics of PHT

Chemically, PHT is a hydantoin derivative (5,5-Diphenylhydantoin) (see fig. 1).

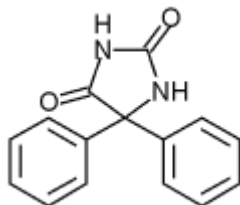


Figure 1: Chemical structure of PHT [12].

PHT is a colour- and odourless powder with a melting point between 292 and 299°C [13]. The substance is an acid with a pKa-value of 8.33 [12]. The chemical characteristics of PHT and PHT-sodium respectively are shown in table 1.

Tab. 1: Chemical characteristics of PHT and PHT-sodium:

Organoleptic characteristics	PHT is a white, crystalline, odourless and flavourless powder. The sodium salt is also white, crystalline, and lightly hygroscopic, with a bitter, soapy flavour [14]
Chemical structure	C ₁₅ H ₁₂ N ₂ O ₂
Molecular weight	252.3 g/mol (PHT) 274.2 g/mol (PHT-sodium)
Solubility	< 20 µg/mL [15].
pKa	8.33 (weak acid) [14]

Pharmacology

Pharmacokinetics

The pharmacokinetic parameters of PHT are shown in table 2 [16].

Table 2: Pharmacokinetic parameters of PHT:

Absolute oral bioavailability	85% [17]
Protein binding	85-95% mostly albumin
Volume of distribution	0.8 L/kg [18]
“Half-life”	30 hours ¹ [19]
Clearance	0.016-0.042 L/kg ^h [14]
Q ₀ (extrarenal eliminated bioavailable dosis-part if normal kidney-function)	1 [19]
Therapeutic range	40-80 µmol/L (10-20 µg/mL)

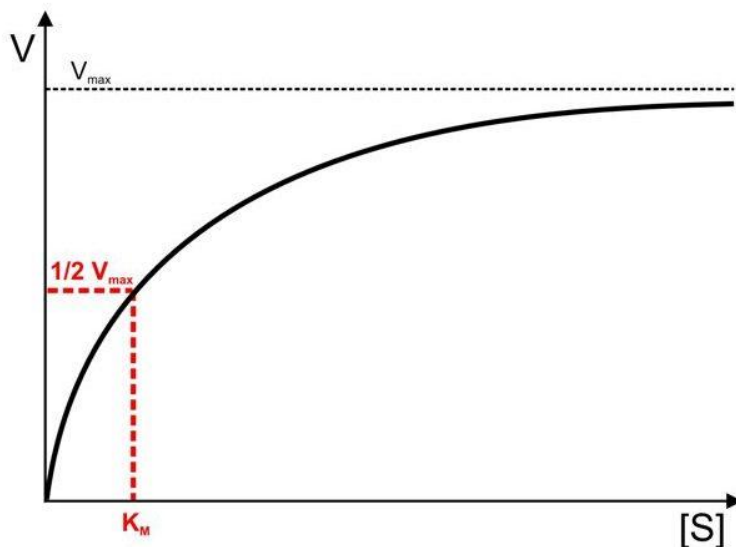
¹ PHT has a nonlinear kinetic; that means the half-life increases with larger doses. Indicated is a typical half-life for therapeutic doses.

Metabolism

PHT is almost completely metabolised in the liver (hepatic oxidation); less than 5% of a dosage is excreted unchanged. The metabolites have no important anticonvulsive effect [20-21]. The responsible enzymes are cytochromes, above all CYP2C9 and CYP2C19. The range of enzyme saturation can be exceeded even in therapeutic dosing, which results in a nonlinear correlation between the dosage and the serum levels [22].

The pharmacokinetics of PHT is already in normal dosages complex because of a non-linear, saturable Michaelis-Menten kinetic and because of the interindividual, genetic differences in the metabolism (see figure 2).

$$v = \frac{v_{\max} \cdot [S]}{K_m + [S]}$$



v = metabolizing ratio [mg/d]

v_{\max} = maximal capacity of the metabolizing enzyme system [mg/d]

K_m = Michaelis-Constant (concentration of the substrate by half enzyme-saturation) [mg/L]

[S] = substrate- and PHT-concentration in the Steady-State respectively [mg/L]

Fig. 2: Michaelis-Menten kinetics. Relation between metabolizing ratio (v) and substrate-concentration $[S]$ of an enzym-katalytic reaction after Michaelis and Menton; shown is the example of PHT. v_{\max} is the maximal capacity of the metabolizing enzyme system. The concentration of the substrate by half enzyme-saturation is called Michaelis-Menten-constant (K_m). Up to this point (6 mg/L) the kinetic is linear. The therapeutic range of PHT is between 10-20 mg/L (40-80 $\mu\text{mol/L}$).

PHT shows a large interindividual variability in v_{\max} and K_m . The values of K_m are 4-6 mg/L and v_{\max} 6-8 mg/kg/day respectively [14].

Total drug concentrations versus free drug concentration

Total blood drug concentration consists of a protein-bound and free (unbound) fraction. Most drugs are bound to serum proteins to a various extent. Only unbound or free drug is pharmacologically active [23].

Normally total drug is measured for therapeutic drug monitoring (TDM), because there is equilibrium between bound and free drugs, and concentration of free drug can be predicted from total drug concentration [23]. Free drug monitoring is not a routine procedure in clinical laboratories due to technical difficulties and lack of established reference ranges for free drugs [23-24]. Furthermore the unbound fraction has to be separated from the bound fraction (e.g. dialysis, ultrafiltration) [23-26]. In general strongly bound protein drugs (> 80%) are candidates for free drug concentration determination.

Free PHT concentration

Traditionally used techniques to determine the free drug concentration are the equilibrium dialysis (microdialysis), or the ultrafiltration (with devices with a cut-off-filter) [24-25, 27].

The free fraction (fF) of a drug is calculated as:

$$fF = \frac{\text{unbound drug concentration}}{\text{total drug concentration}}$$

The fF is the percentage of the whole drug concentration, which is not bound to proteins (e.g. plasma protein binding = 99% \rightarrow $fF = 0.01$) [28].

The therapeutic range for the free PHT concentration in the serum is narrow as well (1-2 mg/L and 4-8 μ mol/L respectively). The critical value for toxicity is > 2 mg/mL. Sometimes the therapeutic range of free PHT is indicated as percentage of total PHT; it should be between 6 and 12% of total PHT [29], which makes the TDM of the fF necessary in variable albumin concentration to make the therapy efficacy and to avoid toxicity. TDM of unbound (free) drugs belongs due to highly technical and time-consuming efforts and costs not to the routinely done measurements in clinical laboratories and therefore requires a rational indication.

Pharmacodynamic

The antiepileptic effect of PHT derives from the blockade of voltage-dependent sodium channels in the neuronal cell-membrane (see fig. 3). Thus, rapid potentials along the axons can be interrupted and repeated unloading can be suppressed [14, 30].

Characteristically for the effect of PHT is the dependence of the action from the opening-probability of a channel (so called use-dependance) [31].

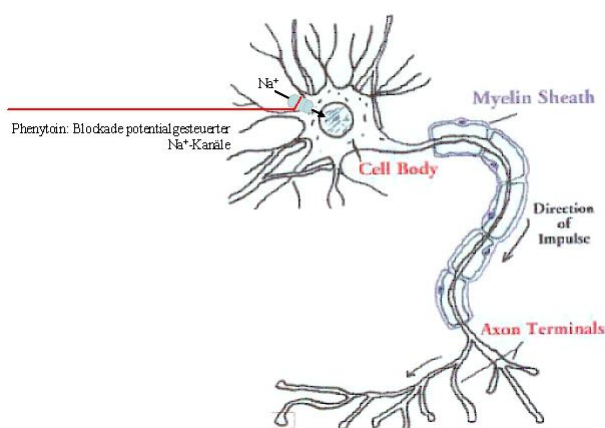


Fig. 3: Schematic mechanism of action of PHT [32].

Therapeutic Drug Monitoring (TDM) of PHT

There are many reasons for a regular TDM while treatment with PHT:

- Correlation between plasma-concentration and reduction of clinical and encephalographic symptoms of epilepsy. [33].
- Because of long-term epilepsy treatment, the specific characteristics and pharmacokinetics of the antiepileptics, the TDM has become invaluable in the optimization of the treatment; a continuing TDM guarantees the efficacy and prevents in the meantime the toxicity of the antiepileptic treatment [34-36].

A good and suitable TDM is dependent on rapid, sensitive, and specific analytical techniques. The control of the individual PHT serum levels and the dose adjustment are nowadays established routinely to maintain an efficacious and compliant long-time therapy. TDM as far as PHT is concerned is very important because its characteristics fulfil TDM requirements:

- narrow therapeutic index
- non-linear, saturable Michaelis-Menten pharmacokinetics
- interindividual, genetic differences in metabolism
- correlation between blood serum level and toxicity
- long-time therapy
- drug-interactions

Bayesian Forecasting

Population data for the calculation of the individual maintenance dose are shown in tab. 3 [37].

Tab. 3: Population data for the calculation of the individual maintenance dose of PHT [37]:

Parameter		Standard deviation	
V_{max}	$32.42 \text{ mg} \cdot (\text{body weight})^{0.6}$ 415 mg/d for a 70 kg weight patient	σ_{Vmax}	20%
K_m	5.7 mg/L	σ_{K_m}	51%
V_d	1 L/kg	σ_{V_d}	23%

The following parameters must be known for the individual dose-calculation:

- body weight of the patient
- dosage as IV bolus or as oral maintenance dose for the appropriate given time-point
- trough level at the given time-point

With this data, the following parameters can be calculated:

- patient parameters (v_{max} , K_m , V_d) \pm 68% confidence interval
- recommended 12 and 24 hours maintenance dose (oral or IV) for reaching the aimed steady-state-level
- prediction of the serum level at the time-point of the next determination

As PHT has a non-linear kinetic, the individual maintenance dose in the Bayesian forecasting program is calculated with a method with integrated Michaelis-Menten-Kinetic [38].

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3 AIMS OF THE THESIS

In a first and most important part of the investigation, a long-term retrospective evaluation was intended on the totality of PHT patients receiving TDM with different support in an acute care teaching hospital. An evaluation of PHT treatment measured by achieving therapeutic PHT serum levels in two groups of patients was compared. In one group physicians and nurses were supported by a round the clock pharmacy service - providing guidelines, teaching and information for a rapid IV loading PHT medication regimen with individual dose calculation using Bayesian Forecasting (BF) algorithms (BF group) after the PHT TDM measurement. In the other group of non-supported patients, only SPC nomogram-related conventional prescribing material was available.

In addition, the influence of patients' variables like gender and age should be investigated and validated in the BF group.

In a second subgroup investigation, the usefulness of the Sheiner-Tozer algorithm was evaluated to calculate the free PHT dose fraction using total PHT serum levels in unselected patients with low albumin (≤ 35 g/L) in the above long-term investigation.

In a third part, the assessment to correlate PHT blood serum levels, with "brain PHT levels" representing the site of action of PHT, available from extracellular fluid from microdialysate in neurosurgical patients, was aimed.

In a first step, a sensitive analytical method with GC-MS was intended to measure (free) PHT in "aqueous" patient samples. A simple and effective sample extraction method had to be included for different biological matrices (blood, dialysates, and saliva). Indicators for the sensitivity had to be calculated [limit of detection (LOD) and limit of quantification (LOQ)]. Reproducibility, suitability of calibration, stability of PHT and matrix effects had to be addressed.

Validation according to International Organization for Standardization (ISO) 17025 in the corresponding investigation with samples from patients (clinical trials) was aimed for the analytical method.

4 OVERALL SUMMARY OF THE THESIS

PHT is one of the mostly used and well established anticonvulsants for the treatment of epilepsy and a standard in the antiepileptic prophylaxis in adults with severe traumatic brain injuries before and after neurosurgical intervention.

Its therapeutic use is challenging as PHT has a narrow therapeutic range and shows non-linear kinetics with a wide interindividual variability in clearance (CL):

$$CL = \frac{V_{max}}{K_m + C}$$

V_{max} (6-8 mg/kg/day), K_m (4-6 mg/L); C: plasma conc [1, 2].

It is extensively metabolized by a variety of CYP enzymes and also transported by genetically variable transporters. PHT shows 85-95% binding to plasma proteins mostly albumin. This renders PHT also an important drug interaction candidate. ADR range from mild to severe and are either dose-dependent or hypersensitivity reactions. Therefore, therapeutic drug monitoring is often required [3]. A rational timing for probe sampling and good interpretation of the lab data translated in optimal individual dosing are necessary. Therapeutic guidance especially in teaching hospitals are needed, have to be implemented and their usefulness if possible assessed.

Bayesian Forecasting (BF) versus conventional dosing (CD): a retrospective, long-term, single centre analysis

In the hospital, medication management for effective antiepileptic therapy with PHT often needs rapid IV loading and subsequent dose adjustment according to TDM. To investigate PHT performance in reaching therapeutic target serum concentration rapidly and in a sustainable manner, a BF regimen was compared to CD, according to the official summary of product characteristics. In a Swiss acute care teaching hospital (Kantonsspital Aarau) serving as a referral centre for neurology and neurosurgery, a retrospective, single centre, and long-term analysis was assessed by using all PHT serum tests from the central lab from 1997 to 2007. The BF regimen consisted of a guided, body weight-adapted rapid IV PHT loading over five days with pre-defined TDM time points. The CD was applied without written guidance.

Assuming non-normally distributed data, non-parametric statistical methods were used. A total of 6'120 PHT serum levels (2'819 BF and 3'301 CD) from 2'589 patients (869 BF and 1'720 CD) were evaluated and compared. 63.6% of the PHT serum levels from the BF group were within the therapeutic range versus only 34.0% in the CD group ($p < 0.0001$). The mean BF serum level was $52.0 \pm 22.1 \mu\text{mol/L}$ (within target range), whereas the mean serum level of the CD was $39.8 \pm 28.2 \mu\text{mol/L}$ (sub-target range). In the BF group, men had small but significantly lower PHT serum levels compared to women ($p < 0.0001$). The CD group showed no significant gender difference ($p = 0.187$). A comparative sub-analysis of age-related groups (children, adolescents, adults, seniors, and elderly) showed significant lower target levels ($p < 0.0001$) for each group in the CD group, compared to BF. Comparing the two groups, BF showed significantly better performance in reaching therapeutic PHT serum levels rapidly and for a longer duration.

Free PHT assessment

However, total serum drug levels of difficult-to-dose drugs like PHT are sometimes insufficient. The knowledge of the free fraction is necessary upon given patient conditions for correct dosing. In a subgroup analysis of the above BF vs. CD study we evaluated the suitability of the Sheiner-Tozer algorithm to calculate the free PHT fraction in hypoalbuminemic patients. Free PHT serum concentrations were calculated from total PHT concentration in hypoalbuminemic patients and compared with the measured free PHT. The patients were separated into two groups (a low albumin group; $35 \leq \text{albumin} \leq 25 \text{ g/L}$ and a very low albumin group; $\text{albumin} < 25 \text{ g/L}$). These two groups were compared and statistically analysed for the calculated and the measured free PHT concentration. The calculated (1.2 mg/L , $\text{SD}=0.7$) and the measured (1.1 mg/L , $\text{SD}=0.5$) free PHT concentration correlated. The mean difference in the low and the very low albumin group was 0.10 mg/L ($\text{SD}=1.4$, $n=11$) and 0.13 mg/L ($\text{SD}=0.24$, $n=12$), respectively. Although the variability of the data could be a bias, no statistically significant difference between the groups was found: t-test ($p=0.78$), the Passing-Bablok regression, the Spearman's rank correlation coefficient of $r=0.907$ and $p=0.00$, and the Bland-Altman plot including the regression analysis between the calculated and the measured value ($M=0.11$, $\text{SD}=0.28$).

Therefore, we concluded that in absence of a free PHT serum concentration measurement also in hypoalbuminemic patients, the Sheiner-Tozer algorithm

represents a useful tool to assist TDM to calculate or control free PHT by using total PHT and the albumin concentration.

GC-MS Analysis of biological PHT samples

To correlate PHT blood serum levels, with “brain PHT levels” representing the site of action of PHT, extracellular fluid from microdialysates in neurosurgical critically ill (ICU) patients could be analyzed for PHT by an appropriate quantifying analytical method, qualified and validated to be used in a clinical trial. In this investigation we describe the development and validation of a sensitive gas chromatography–mass spectrometry (GC–MS) method to identify and quantitate PHT in brain microdialysate, saliva and blood from human samples. For sample clean-up a SPE was performed with a nonpolar C8-SCX column. The eluate was evaporated with nitrogen (50°C) and derivatized with trimethylsulfonium hydroxide before GC-MS analysis. 5-(p-methylphenyl)-5-phenylhydantoin was used as internal standard. The MS was run in scan mode and the identification was made with three ion fragment masses. All peaks were identified with MassLib. Spiked PHT samples showed recovery after SPE of $\geq 94\%$. The calibration curve (PHT 50 to 1'200 ng/ml, n=6 at six concentration levels) showed good linearity and correlation ($r^2 > 0.998$). The limit of detection was 15 ng/mL, the limit of quantification was 50 ng/mL. Dried extracted samples were stable within a 15% deviation range for ≥ 4 weeks at room temperature. The method met International Organization for Standardization standards and was able to detect and quantify PHT in different biological matrices and patient samples. The GC-MS method with SPE is specific, sensitive, robust and well reproducible and therefore, an appropriate candidate for pharmacokinetic assessment of PHT concentrations in different biological samples of treated patients.

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5 METHODS, RESULTS AND DISCUSSION

The thesis is based on three accepted, PubMed cited peer-reviewed publications. The full text papers are included in the text.

The first publication refers to the retrospective analysis of Bayesian forecasting compared to conventional dosing of intravenous phenytoin. The second publication refers to the value of the Sheiner Tozer equation to calculate the free phenytoin level assessed in patients of the first study with measured free phenytoin levels. And the third publication refers to the analytical part of the thesis with the development and validation of a sensitive and reliable GC-MS method including clean-up for liquid patients' sample to determine PHT.

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Intravenous phenytoin: a retrospective analysis of Bayesian forecasting versus conventional dosing in patients

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Abstract *Background* In the hospital, medication management for effective antiepileptic therapy with phenytoin (PHT) often needs rapid IV loading and subsequent dose adjustment according to therapeutic drug monitoring (TDM). *Objective* To investigate PHT performance in reaching therapeutic target serum concentration rapidly and sustainably, a Bayesian forecasting (BF) regimen was compared to conventional dosing (CD), according to the official summary of product characteristics. *Setting* A 500–600 bed acute care teaching hospital in Switzerland, serving as a referral centre for neurology and neurosurgery. *Method* In a retrospective, single centre, long-term analysis of hospitalized in- and out-patients, all PHT serum tests from the central hospital laboratory from 1997 to 2007 were assessed. The BF regimen consisted of a guided, body weight-adapted rapid IV PHT loading over 5 days with pre-defined TDM time points. The conventional dosage was performed without written guidance. Assuming non-normally distributed data, non-parametric statistical methods for analysis were applied. *Main outcome measure* The extent of target therapeutic PHT serum levels (40–80 $\mu\text{mol/L}$) was measured and compared between the two regimens. Also, the influence of gender and age was analysed. *Results* A total of 6,120 PHT serum levels (2,819 BF and 3,301 conventionally dosed) from 2,589 patients (869 BF and 1,720 conventionally dosed) were evaluated and compared. 63.6 % of the PHT serum levels from the BF group were within the therapeutic range, compared with

only 34.0 % in the conventional group ($p < 0.0001$). The mean BF serum level was $52.0 \pm 22.1 \mu\text{mol/L}$ (within target range) ($n = 2,819$), whereas the mean serum level of the CD was $39.8 \pm 28.2 \mu\text{mol/L}$ (sub-target range) ($n = 3,301$). In the BF group, men had small but significantly lower PHT serum levels compared to women ($p < 0.0001$). The conventionally dosed group showed no significant gender differences ($p = 0.187$). A comparative sub-analysis of age-related groups (children, adolescents, adults, seniors, and elderly) showed significantly lower target levels ($p < 0.0001$) for each group in the conventional dosed group, compared to BF. *Conclusion* Comparing the two cohorts, BF with the well-defined dose regimen showed significantly better performance in reaching therapeutic PHT serum levels rapidly and for longer duration.

Keywords Bayesian forecasting · Conventional dosing · Hospital population · Medication management · Phenytoin · Therapeutic drug monitoring

Impact of findings on practice statements

- Good medication management with TDM requires a multidisciplinary team approach.
- To give support and to take leadership for guidance in handling, TDM, and dose adaptation of critical dose drugs, appropriately trained pharmacy staff must be accessible around the clock.
- Successful and sustainable adherence to therapeutic guidelines relies on continuous use of a variety of complementary tools (orally and written instructions, personal intervention on the ward, data collection and reporting).

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Introduction

Phenytoin (PHT) is a standard, effective and widely used drug prescribed to prevent, control and treat epileptic seizures. Although PHT use as an anticonvulsant in hospitals is diminishing due to its complex pharmacokinetics, it is almost the only drug authorized for seizure prevention e.g. in neurosurgery (on-label use). PHT has been shown to decrease the risk of post-traumatic seizures, before and after neurosurgery in adults, and to prevent early seizures after acute, traumatic brain injuries [1–4].

PHT's non-linear, saturable pharmacokinetics and narrow therapeutic index (40–80 $\mu\text{mol/L}$ and 10–20 mg/L, respectively in serum), requires therapeutic drug monitoring (TDM) for effective and safe usage. The established relationship between serum concentration and toxic/therapeutic effect and the inter-individual dosage variation due to variable metabolism and drug-interactions are further grounds for a TDM. PHT is available as a parenteral formulation enabling rapid IV loading which is often required but difficult to manage properly [5–10]. The PHT summary of product characteristics (SPC) provides dosing nomograms, but IT-supported forecasting programs also exist [11–13]. Bayesian forecasting (BF) guided PHT dosing is well-established and its clinical utility is documented [14–20].

In 1994, a rapid phenytoin IV loading regimen based on BF was established [21]. Accompanied by a pharmacy TDM support service it was introduced throughout the study centre in 1997. The conventional dosing (CD) according the product information leaflet was still in use. The main indications for PHT treatment were the pre- and post-operative prophylaxis of seizures e.g. in brain tumour surgery, in patients with brain oedema or brain trauma, and in the management of persistent seizures (status epilepticus). The BF regimen consisted of a body weight-adapted

loading dose, followed by a standardized maintenance dose period, with subsequent individually calculated maintenance doses of PHT serum trough levels, according to a protocol (see “Methods” section and Fig. 1). A BF program assuming non-normally distributed population data was introduced in which each non-steady-state serum trough level contributes to predict the steady-state serum concentration levels. The patient's individual pharmacokinetic parameters K_m , v_{max} , and V_d were calculated in an iteration process from the individual blood level measurements and from the initial population kinetic values of a general hospital patient group mostly with poly-pharmaco-therapy and varying age and body weight [22].

Aim of the study

The aim of the present investigation was to compare and validate the attainment of therapeutic PHT serum levels, between a pharmaceutically supported rapid IV loading PHT medication management using BF algorithms with non-supported SPC nomogram-related conventional prescribing, in an acute care teaching hospital. The influence of the variables gender and age should also be assessed.

Method

Patients and data recording

Patients' data from more than 10 years (1997–2007), was retrospectively analyzed. A total of 6,120 PHT serum levels (2,819 BF and 3,301 CD) from 2,589 patients (869 BF and 1,720 CD) were compared. Most patients came from neurology and neurosurgery, explaining the high number of

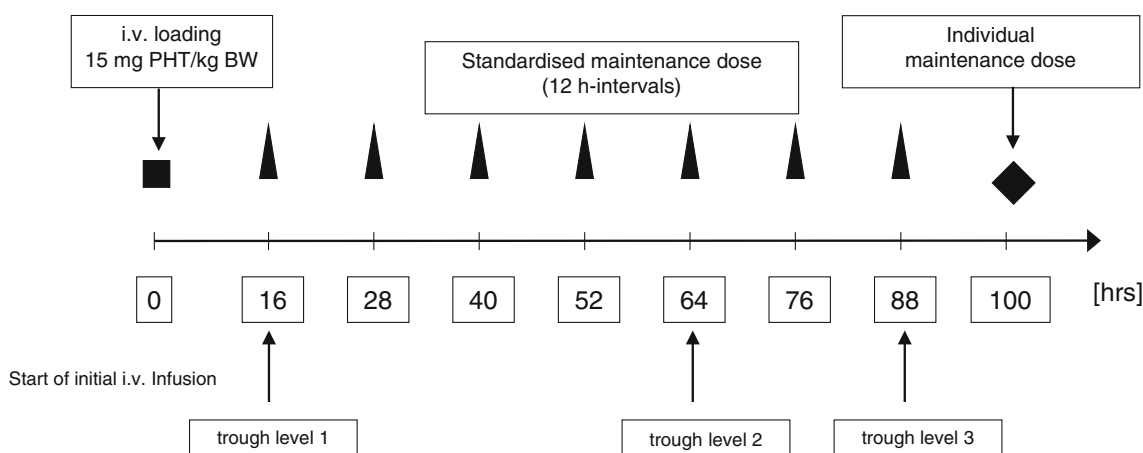


Fig. 1 Bayesian dosing loading regimen. Loading dose: 15 mg/kg body weight (BW) phenytoin. Standardised maintenance doses: given every 12 h for 4–5 days, subsequently, an individually maintenance

dose was calculated. Trough levels were measured at 16, 64 h (76 h resp.) and 88 h (100 h resp.) after starting the therapy

patients in this study; the two cohorts were comparable in the number of serum values and indication for IV PHT treatment. For age-related sub-analyses, patients were assigned to five groups (see Table 1). The BF patients' data were more comprehensive; they were recorded in a pharmacy database which included the patient's initials, gender, age, body weight, PHT (trough) serum levels (in $\mu\text{mol/L}$), the starting time of the initial loading dose, the time points of PHT serum level (concentration) determination, the dose, and TDM time point recommendations including level forecast. Patients were encoded with an identification number allowing anonymous data analysis. For CD patients, only initials, gender, age, values of PHT (trough) levels ($\mu\text{mol/L}$), and date of the PHT serum level determination were accessible from the laboratory's order forms. All PHT-treated patients not registered in the BF records, were assumed to be dosed conventionally.

Study design

The retrospective, open design, single center analysis was carried out in a Swiss 500–600 bed acute care teaching hospital providing internal medicine, general and specialized surgery, pediatrics, obstetrics and gynecology for 500,000–600,000 inhabitants. It served as a neurology and neurosurgery referral center.

Treatment protocols

The CD group was dosed according to the manufacturer's SPC (Epanutin[®] PHT-Na, Pfizer, Zürich, Switzerland; authorized in Switzerland till 2006 or Phenhydan[®] PHT, Desitin Pharma GmbH, Liestal, Switzerland) [11, 12]. The initial daily loading dose was four times 250 mg PHT sodium (a total equivalent to 920 mg PHT) or PHT (depending on the selected formulation). The individual PHT IV doses were infused every 6 h in 100 mL normal saline over 15 min. The daily maintenance IV or p.o. dose was three times 100 mg PHT sodium (equivalent to

276 mg PHT) or PHT. The oral dose was not corrected for PHT's bioavailability of 85 %. TDM serum (trough) levels determination requests were at the physician's discretion. There was no pharmacy TDM support for the CD cohort.

Bayesian forecasting

The BF patients were treated according to the regimen in Fig. 1, implemented and managed by the pharmacy, with repetitive detailed written and oral instructions for physicians and nurses. The initial loading dose of 15 mg PHT/kg body weight was diluted in 100, 250 or 500 mL normal saline in a concentration ≤ 7.5 mg PHT/ml; 7.5 mg PHT acid/ml saline was shown not to precipitate in a microscopic solubility test (data not shown). The loading dose was infused over 4 h into a large central or peripheral vein using a ≥ 20 gauge catheter for optimal tolerance. Standard IV maintenance doses were administered over 15 min every 12 h from day 1 to day 5; twice 175 mg PHT for ≤ 70 kg body weight and twice 202 mg PHT for >70 kg body weight patients, respectively. Exceptional standard maintenance doses of 225 mg PHT and 250 mg PHT for patients ≤ 70 kg body weight and >70 kg, respectively were given p.o. every 12 h, compensating for the 85 % bioavailability.

An individually calculated maintenance dose was proposed starting from day 5, together with a recommendation for the next TDM and serum level forecast. The calculation required the patient's body weight and the (three) TDM levels; the 1st before the first maintenance dose (16 h after starting the therapy), the 2nd before the morning dose on day 4 (64 or 76 h after starting the therapy), and the 3rd on day 5 (88 or 100 h after starting the therapy) (Fig. 1). The calculation used a Turbo Pascal 6.0 written program and allowed a non-steady-state approximation by iteration steps [13]. A steady-state PHT serum concentration of 44 $\mu\text{mol/L}$ (11 mg/L) was targeted. Initial population data from S. Vozeh and Th. Grasela were used [V_d (1 L/kg), K_m (5.7 mg/L) and v_{\max} ($32.42 \text{ mg} \times (\text{body weight})^{0.6}$)] [9–13, 21, 22]. The program calculated the patient's individual parameters for v_{\max} , K_m , and V_d (with the 95 % CI), and were filled in the TDM reporting form for the ward [13].

Treating physicians decided on the treatment protocol; it was influenced by the pharmacy's BF initiative. There was no specific bias detected in the adherence to the different protocols in the two groups.

Outcome, data analysis and statistics

All PHT levels were determined using a homogenous enzyme immune test (EMIT, Syva corporation (Siemens Medical Devices)) [13]. All PHT serum concentrations assessed in the central hospital laboratory were faxed to the

Table 1 Population (number of phenytoin serum levels in the different age groups), $n = 6,120$

Age class	Bayesian forecasting (BF)	Conventional dosing (CD)	Total
<16 years old (children)	21	138	159
16–20 years old (adolescents)	70	60	130
21–65 years old (adults)	1,652	1,988	3,640
66–80 years old (seniors)	909	892	1,801
>80 years old (elderly)	167	223	390
Total	2,819	3,301	6,120

hospital pharmacy. All values calculated and compared referred to PHT acid.

The primary purpose of the study was to compare the resulting PHT serum (trough) levels with the intended ones: primarily the proximity of PHT levels to the BF target value of 44 $\mu\text{mol/L}$ and secondly, the aim to achieve a therapeutic PHT serum level range of 40–80 $\mu\text{mol/L}$. The results were assessed for gender and age variables. The investigation served as validation of the BF method, compared to the conventional regimen (CD) in a non-selected cohort of hospital patients (no exclusion criteria).

The STATA-program (Stata Corp., Version 10, www.stata.com) was used for statistics. Non-normally distributed data was assumed and therefore, non-parametric tests applied. Pearson's χ^2 distribution test and Fisher's Exact test (for small samples) were used for statistical significance analyses, such as the distribution of PHT serum levels in sub-target, target, and super-target ranges. The Mann–Whitney U-test was used for the gender homogeneity check and the Kruskal–Wallis-test for variance analysis of patient's age on measured PHT serum levels.

The box-plot presentation included mean, median and 25–75 interquartile ranges (IQR) of data; outliers were defined as ≥ 1.5 times outside the IQR.

Ethical committee information

The retrospective analysis aimed to validate the BF PHT IV loading regimen by comparing it with a SPC nomogram-based CD, and had no influence on the PHT therapy of the subjects investigated. There was no access to the patients' charts. Therefore, upon submission of the investigation to the local Ethics Committee, ethical approval was deemed unnecessary.

Results

There was a significant difference between the two groups in the number of serum levels within the target range ($p < 0.0001$). In the BF group 63.6 % of the levels were in the target range and 27.5 % below, while the CD group had only 34 % in target and 57.7 % below (Table 2).

Table 2 Distribution of phenytoin serum levels (n = 6,120): Bayesian forecasting group (n = 2,819) compared to conventional dosing (n = 3,301)

Group	Number of phenytoin serum levels (absolute and relative no.)			Total
	<40 $\mu\text{mol/L}$	40–80 $\mu\text{mol/L}$	>80 $\mu\text{mol/L}$	
Bayesian forecasting	775 (27.5 %)	1,793 (63.6 %)	251 (8.9 %)	2,819 (100.0 %)
Conventional dosing	1,904 (57.7 %)	1,122 (34.0 %)	275 (8.3 %)	3,301 (100.0 %)
Total	2,679 (43.8 %)	2,915 (47.6 %)	526 (8.6 %)	6,120 (100.0 %)

Relative values are given in brackets

During the entire observation period, the BF was significantly more effective in achieving target serum levels compared to CD ($p < 0.0001$, n = 6,120), (Fig. 2). The mean \pm SD BF serum level was $52.0 \pm 22.1 \mu\text{mol/L}$ (in target) (n = 2,819) and exceeded the defined target level of 44 μM , while the respective values for CD were $39.8 \pm 28.2 \mu\text{mol/L}$ (below target) (n = 3,301). The maximal (toxic) value was 232 $\mu\text{mol/L}$ in the CD group, compared to 169 $\mu\text{mol/L}$ in the BF group.

Gender analysis

In the BF group, men had minor but significant lower PHT serum levels compared to women ($p < 0.0001$), the mean and median were always in the therapeutic range: women (mean 53.0 $\mu\text{mol/L}$, median 53.2 $\mu\text{mol/L}$, n = 1,345) and men (mean 51.0 $\mu\text{mol/L}$, median 50.8 $\mu\text{mol/L}$, n = 1,474). The CD group showed no gender differences ($p = 0.187$); the values for women: mean 40.6 $\mu\text{mol/L}$, median 36.3 $\mu\text{mol/L}$, n = 1,312; for men: mean 39.3 $\mu\text{mol/L}$, median 32.7 $\mu\text{mol/L}$, n = 1,989.

Analysis of age

The overall age characteristics were comparable, for BF: min 7.6 years, mean 57.6 years, max 92.1 years and for the CD group: min 1.5 years, mean 54.8 years, max 100.0 years. A comparative sub-analysis of the age-related groups (children, adolescents, adults, seniors, and elderly) showed significant lower target levels ($p < 0.0001$) for each group in the CD group compared to BF (Fig. 3).

BF validation (TDM support)

Table 3 shows the relative extent of the initially reached target levels in the loading phase (trough levels 1–3) for BF.

The proportion of PHT TDM levels using BF ranged from 16.7 % (2007) to 80.3 % (2004), and from 19.7 % (2004) to 83.3 % (2007) using CD (Fig. 4). The relative annual proportion of BF and CD patients varied from 1:0.2 to 1:4.07.

Fig. 2 Box plot of mean annual phenytoin serum levels for Bayesian forecast (BF) and conventional dosing (CD) from 1997 to 2007 ($n = 6,120$). The therapeutic serum level range for phenytoin (40–80 $\mu\text{mol/L}$) is marked by the red lines. Outliers are represented by dots (max. values). (Color figure online)

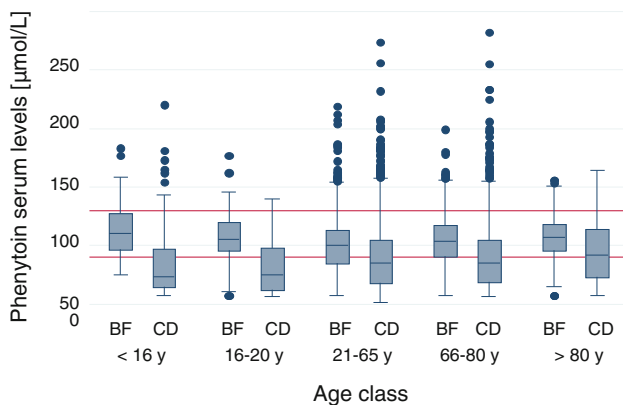
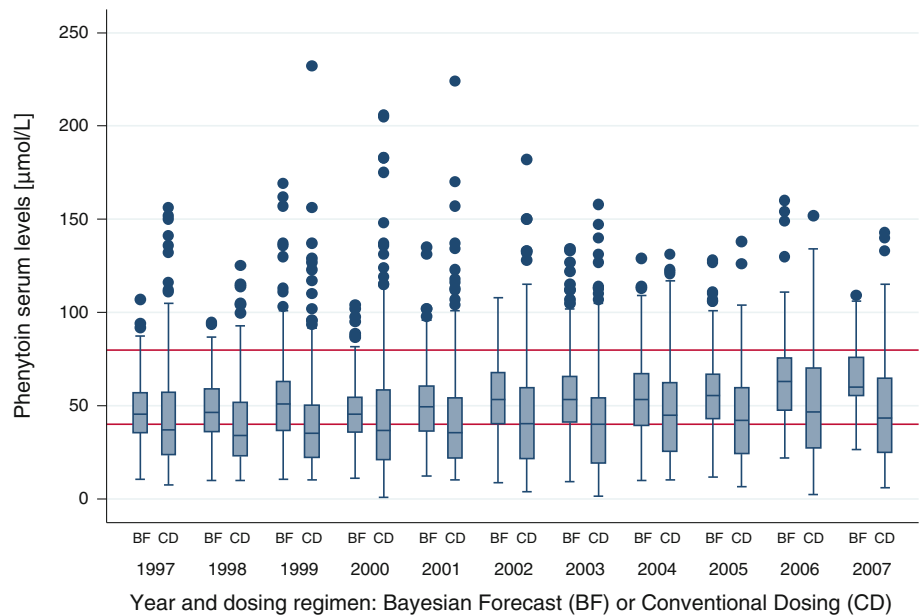


Fig. 3 Phenytoin serum level and age ($n = 6,120$). Box plots show the phenytoin serum levels of the Bayesian forecast group (BF) (left) and of the conventional dosing group (CD) (right). The therapeutic range (40–80 $\mu\text{mol/L}$) is between the red lines. Dots signify outliers. (Color figure online)

Discussion

PHT TDM is well-established to control effectiveness and toxicity in patients but interpretation of the data and dose adaptation is difficult. Resulting medication errors are of special concern in a teaching hospital where there are often changes in staff and varying levels of experience. As a consequence, appropriate medication management guidance is of great importance.

The analysis of more than 6,000 PHT serum measurements showed significant superiority in achieving therapeutic serum levels quickly (Tables 2, 3; Fig. 2) for the pharmacy-supported BF PHT protocol compared to the conventional, SPC-related therapeutic procedure (CD), managed only by the prescribing physician. With the BF

protocol, the 40–80 μM target level was reached rapidly in almost 80 % of patients after the first dose and two-thirds remained there at the end of the initial standardized loading phase (Table 3). The superior PHT target level achievement indicates better control of dose, V_d (body weight), clearance, and TDM interpretation, although no exclusion of patients for age or potentially interacting co-medications was carried out. This proves the therapeutic appropriateness of the BF protocol and its safety in not exposing patients to toxic PHT doses in a general hospital population (Figs. 2, 3). The study also validates the BF protocol as opposed to the standard, nomogram-deviated CD.

Compared to CD, the superiority of BF also results from a higher, body weight-adapted PHT loading regimen, namely a first loading dose of 15 mg PHT/kg, followed by a body weight-adapted standard maintenance dose. The different IV PHT administration times—BF 4 h for the initial loading dose versus 15 min for CD with an initial dose of 250 mg—resulted in a significantly smaller infusion rate (mg PHT/kg/min) for BF, explaining the excellent tolerance of the higher BF dose. In contrast to CD, the pharmacy TDM service for BF included a proposal for dose and TDM timing, based on population data calculation, reducing also the number of meaningless PHT serum tests [21]. The BF regimen using Bayesian algorithms and population kinetic parameters allowed for individual calculation of a non-steady state dose with even one single TDM measurement.

Influence of gender and age

In the BF group, men had statistically significant lower PHT serum levels compared to women ($p < 0.0001$),

Table 3 Drug concentration range of Bayesian forecasting in the 5 days IV loading phase: relative distribution of phenytoin trough levels 1–3 (day 1–5), in %; n = 1,800

Drug concentration range	Serum trough level 1 (16 h after start of therapy)	Serum trough level 2 (64 and 76 h resp. after start of therapy)	Serum trough level 3 (88 and 100 h resp. after start of therapy)
<40 µmol/L	17.3	17.7	21.3
40–80 µmol/L	78.6	72.1	66.1
>80 µmol/L	4.1	10.2	12.6

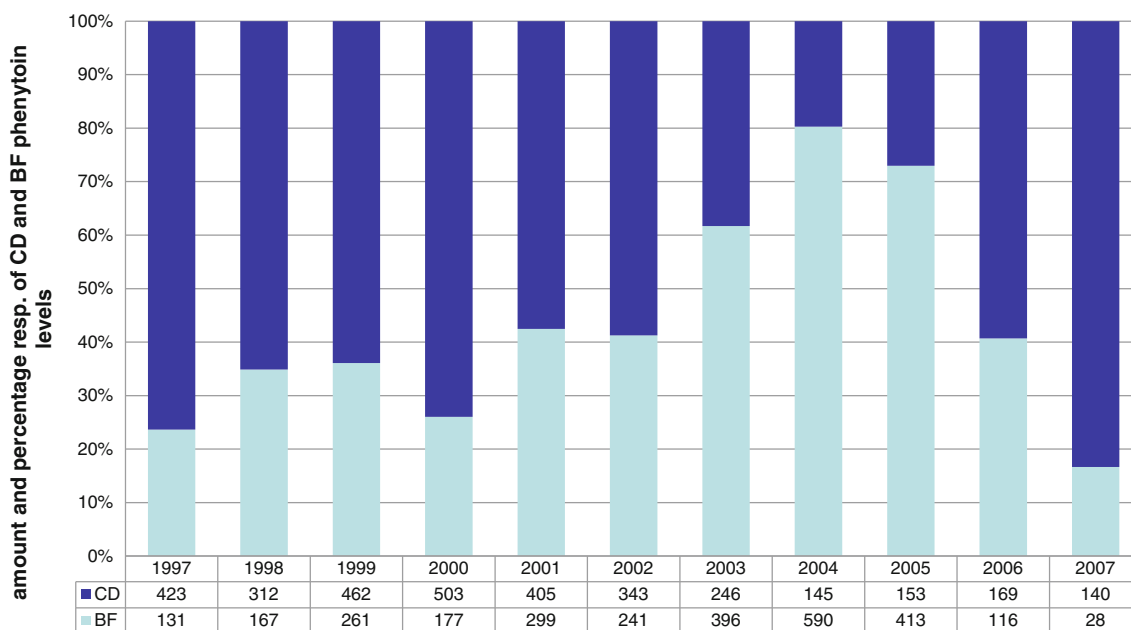


Fig. 4 Phenytoin serum levels per year and treatment group (n = 6,120). The bars show the annual proportion of the phenytoin serum levels of the Bayesian forecasting group (BF) and of the conventional dosing group (CD) over study period

which may be explained by a slightly different, gender-dependent V_d (body composition, with higher fat mass in women). Based on this study, the gender differences are minimal and not likely to be relevant for therapy success. Therefore, BF can be used gender-independently.

The incidence of epileptic seizures and need for therapy increases in people over 60 years of age, which is also reflected in the patients investigated [23]. Literature is inconsistent on the influence of age on PHT pharmacokinetics [23–27]. The elderly may have lower albumin concentrations (decreased plasma protein binding changes V_d) and reduced renal drug clearance. Children, another sensitive population group, show different pharmacokinetics compared to adults [28]. However, studies were mostly carried out with small numbers of patients, providing limited results. In this large study, the analysis of five different age groups revealed the superiority of BF in reaching target PHT levels, especially in patients below 20 and over 60 years of age. In the two largest groups of adults and seniors, the CD showed a wider variation of PHT serum

levels and more outliers compared to the BF group (Fig. 3). Age-related differences only occurred in the CD group; a possible explanation could be the wider age range (min 1.5 years, max 100 years) compared to BF (7.7–92.1 years) and the lower body weight-adjusted loading dose in CD. In contrast, relevant age-related parameters such as V_d and v_{max} were included in the forecasting calculation of BF. The trend to under-dose patients in the CD group disappeared with increasing age of the patients. It remains to be investigated if prescribers are more cautious in dosing the young in absence of appropriate guidelines.

A general hospital population includes neurological patients needing antiepileptic PHT treatment, or prevention in emergency. Such patients often get multiple, potentially interfering drugs also making it difficult to observe the anti-epileptic effect, e.g. in sedated ICU patients. Therefore, a clinical symptom-independent, lab-based PHT concentration measurement is a reliable outcome parameter. This is especially valid when investigating data from a large sample pool over a long time period, which contrasts

with the small and short-term studies comparing different PHT dosing regimens to date [8, 14–20, 29].

A retrospective, open design was the only possibility and provides a realistic picture of a non-study influenced hospital situation. Nevertheless, it must be stated that a limitation of the study design is the evaluation of lab data without clinical assessment for effectiveness. Although it is a suitable therapy marker not dependent on individual medical assessments, expertise and skills, PHT TDM cannot replace clinical outcome data [30].

The lab values included the total of all PHT serum levels measured over more than 10 years. The extensive, laborious statistical analysis of all the available variables, partially missing in the incompletely defined control group (no chart analysis) was time consuming, also delaying publication. Significant bias was likely introduced by the freedom of regimen choice and the monitoring options at the discretion of the treating physician in the CD group. A significant number of the clinicians, and certain departments did not adhere to the BF protocol, indicating the need for continuous instruction and follow-up of medication guidance.

When comparing this investigation on more than 2,500 patients over 10 years with an initial study done in the same setting on a much smaller number of roughly 500 patients over 2 years, a remarkable shift from CD to BF occurred; from 2.6:1 to 1.17:1 [21]. The increase in BF patients mirrored the importance of steadily instruction, follow-up and the multimodal and multidisciplinary approach for medication management/therapeutic guidelines implementation [31]. Pharmacy support was key to adherence to the BF protocol. From 1997 to 2004 (after introduction and active promotion of the BF protocol by the pharmacy with continuous and compulsory instruction and support), the annual proportion of CD patients dropped from 76 to 20 %. After a change in the pharmacy management in 2005 lead to lack of promotion of the BF PHT protocol, CD increased again to 83 % in 2007 (Fig. 4).

Conclusion

IV loading with PHT for antiepileptic use is complicated, requiring validated therapeutic guidance for successful medication management of this critical dose drug and its TDM. Nowadays, these characteristics diminish the importance of PHT.

Compared to CD, according to SPC, BF with a well-defined PHT protocol is superior and appropriate for initial effective and safe IV dosing and efficient TDM, in a general hospital population.

Already (one to) three non-steady-state serum levels allow for appropriate calculation of an individual dosage

after the initial standardized, effective 5-day PHT loading phase in patients.

A hospital pharmacy service for TDM and the related medication management allows for successful multidisciplinary implementation and follow-up of therapeutic guidelines when continuous support and accessibility are provided.

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Conflicts of interest The submitting author is a scientific director at Vifor Pharma Ltd., Glattbrugg, Switzerland and holds an additional appointment at the Medical Faculty and the Dept. of Pharmaceutical Sciences at the University of Basel, with a professorship in pharmacology and hospital pharmacy. Vifor Pharma Ltd. is not involved in this investigation and has no pharmaceutical products, services or R&D related to the topic. Their focus is on iron deficiency treatment especially with IV iron preparations.

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Free phenytoin assessment in patients: measured versus calculated blood serum levels

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Abstract *Background* Total serum drug levels are routinely determined for the therapeutic drug monitoring of selected, difficult-to-dose drugs. For some of these drugs, however, knowledge of the free fraction is necessary to adapt correct dosing. Phenytoin, with its non-linear pharmacokinetics, >90 % albumin binding and slow elimination rate, is such a drug requiring individualization in patients, especially if rapid intravenous loading and subsequent dose adaptation is needed. In a prior long-term investigation, we showed the excellent performance of pharmacy-assisted Bayesian forecasting support for optimal dosing in hospitalized patients treated with phenytoin. In a subgroup analysis, we evaluated the suitability of the Sheiner-Tozer algorithm to calculate the free phenytoin fraction in hypoalbuminemic patients. *Objective* To test the usefulness of the Sheiner-Tozer algorithm for the correct estimation of the free phenytoin concentrations in hospitalized patients. *Setting* A Swiss tertiary care hospital. *Method* Free phenytoin plasma concentration was calculated from total phenytoin concentration in hypoalbuminemic patients and compared with the measured free phenytoin. The patients were separated into a low ($35 \leq \text{albumin} \leq 25 \text{ g/L}$) and a very low group (albumin $<25 \text{ g/L}$) for comparing and statistically analyzing the calculated and the measured free phenytoin concentration.

Main outcome measures Calculated and the measured free phenytoin concentration. *Results* The calculated (1.2 mg/L (SD = 0.7) and the measured (1.1 mg/L (SD = 0.5) free phenytoin concentration correlated. The mean difference in the low and the very low albumin group was: 0.10 mg/L (SD = 1.4) (n = 11) and 0.13 mg/L (SD = 0.24) (n = 12), respectively. Although the variability of the data could be a bias, no statistically significant difference between the groups was found: *t* test ($p = 0.78$), the Passing–Bablok regression, the Spearman's rank correlation coefficient of $r = 0.907$ and $p = 0.00$. The Bland–Altman plot including the regression analysis revealed no systematic differences between the calculated and the measured value [$M = 0.11$ (SD = 0.28)]. *Conclusion* In absence of a free phenytoin plasma concentration measurement also in hypoalbuminemic patients, the Sheiner–Tozer algorithm represents a useful tool to assist therapeutic monitoring to calculate or control free phenytoin by using total phenytoin and the albumin concentration.

Keywords Phenytoin · Serum concentrations · Sheiner–Tozer equation · Therapeutic Drug Monitoring (TDM)

Impact on practice

- The Sheiner–Tozer algorithm can be successfully used to calculate a missing free phenytoin plasma concentration using the total phenytoin and the albumin plasma levels in hypoalbuminemic patients.
- The Sheiner–Tozer algorithm represents a useful and shortly available calculation tool to assist Therapeutic Drug Monitoring and appropriate dose adjustment of a critical dose drug based on the free dose fraction, e.g.

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the antiepileptic phenytoin and other highly albumin-bound narrow therapeutic index drugs.

- The Sheiner-Tozer algorithm can be successfully implemented for dose checking to reduce and manage medication errors in critical dose drugs.

Introduction

Phenytoin (PHT) is a well-known antiepileptic drug which has also shown effectiveness in post-traumatic surgery [1]. Its targeted therapeutic concentration range in blood serum for adults and children older than three months is 10–20 mg/L (40–80 μ mol/L) [2]. PHT shows 90 % plasma protein binding corresponding to 1–2 mg/L for free PHT in the therapeutic range. Due to its complex, non-linear and highly variable pharmacokinetics, and its significant dose-related toxicity, determining the correct dosage of this drug is difficult and of critical importance for hospitalized [intensive care unit (ICU)] patients. Doses higher than 20 mg/kg in adults consistently result in clinical toxicity. Additionally, the normal lower dosage of 6 mg/kg body weight in adults, or conventional rapid drug loading, frequently leads to over or under dosing [2, 3]. Defining the correct dose of PHT is challenging due to its narrow therapeutic index, highly variable, dose-dependent, non-linear kinetics (with elimination “half-lives” ranging from 16 to 60 h), and the significant risk of drug interactions. This highlights the importance of rapidly available Therapeutic Drug Monitoring (TDM) for total and in selected patients-free PHT serum concentrations in order to facilitate appropriate and safe adjustment of the medication [4–7].

PHT fulfills the prerequisites for a useful TDM with its narrow therapeutic index, high dose variability and most importantly, the existing correlation between drug plasma level and drug efficacy or toxicity. Lab testing for total PHT in serum is routinely available in larger hospitals. In most cases, total drug concentration in blood/plasma is appropriate for TDM; except when the relative concentration in plasma is either too low (<50 %), or highly variable [8]. The total concentration of drugs is easier to determine compared to the unbound fraction, as validated methods for the latter are only available in a few labs. In addition, tests used to analyze total drug levels in plasma or serum are less costly and time consuming than special analyses such as free PHT assessment [8–10]. The concentration of the free drug fraction is useful or required for the TDM of drugs with narrow therapeutic index and for those with highly variable metabolism rates affected by the patient’s genetically-determined enzyme patterns. It is also useful for medications with >90 % plasma protein binding and variable binding plasma protein concentrations such as for

example PHT in the presence of low plasma albumin levels.

The Sheiner-Tozer formula has been proposed to help estimate the free PHT fraction [6]. Total PHT and the serum albumin level must be known for this calculation. As indicated above, hypo-albuminaemia has a significant impact on PHT plasma protein binding and, in the end, only the free available fraction diffuses into tissue or organs like the brain.

Albumin concentration is influenced by diseases such as uremia, decreased kidney function or chronic liver diseases [8]. Albumin might also be low in elderly patients, burn victims and/or critically ill patients, or pregnant women. This translates into variable amounts of bound and unbound PHT fractions as compared to a “standard” patient [11, 12]. A reduced bound fraction of PHT might also result from interacting co-medications that are competing for the same drug protein binding site in serum. Acidic, highly protein-bound drugs as well as free fatty acids are able to force PHT from the plasma protein binding sites, resulting in higher (toxic) free PHT concentrations without changing total PHT concentration [8, 9, 13, 14]. The Sheiner-Tozer algorithm has been designated to determine the free drug fraction (Fig. 1). There are conflicting reports regarding the value of calculated free PHT levels in patients. To further evaluate the Sheiner-Tozer formula in clinical practice, we performed a sub-analysis of our large, long-term PHT single center study on an unselected group of hospitalized low albumin patients treated with PHT, whose total and free PHT values were also available [15].

Aim of the study

This investigation evaluated the usefulness of the Sheiner-Tozer algorithm (Fig. 1) to calculate the free PHT dose fraction using total PHT serum levels in unselected patients with low albumin (<35 g/L) in a hospital setting.

$$\text{Calculated free PHT} = \frac{\text{Total PHT concentration} \times 0.1}{0.9 \times \left(\frac{\text{Albumin concentration}}{44} \right) + 0.1}$$

Fig. 1 The Sheiner-Tozer formula: the calculated free PHT (dphCF) [g/L] is determined with the knowledge of the total PHT (dphT) [mg/L] and the serum albumin value of the patient. 0.9 is the protein bound PHT fraction (90 %) and 0.1 is the free PHT fraction (10 %). 44 is the mean serum albumin value [g/L]

Ethical approval

Additional ethical approval of this retrospective subanalysis of the previously published and approved investigation by the local ethics committee was deemed unnecessary, as the investigation had no influence on the phenytoine therapy or outcome of the anonymously investigated patients.

Method

This subanalysis was carried out as a study cohort collected over more than 10 years and included all patients in a tertiary care hospital who had undergone PHT serum concentration testing. They were investigated on the value of Bayesian PHT dose forecasting. Those patients whose free PHT calculated concentration could be compared with measured levels were selected [15]. All patients with a serum albumin concentration of ≤ 35 g/L (hypo-albuminaemia) who had both total and free PHT serum concentration measurements were included. Of the initial 2500 patient cohort, 23 fulfilled these criteria. Their age ranged from 7 to 86 years; 8 patients were male and 15 female. The patients were hospitalized on different wards: ICU $n = 3$; dialysis $n = 2$; surgery $n = 4$; internal medicine $n = 10$; pediatrics $n = 1$; neurosurgery $n = 2$; orthopedic $n = 1$. The patients were separated into two groups: the low group ($35 \leq \text{albumin} \leq 25$ g/L, $n = 11$), and the very low group (albumin < 25 g/L, $n = 12$).

Total PHT in serum (dphT) was determined using a homogenous enzyme immune test, EMIT, Syva corporation (Siemens Medical Devices) [16]. The immuno assay produced the following precision values: Level 1: 7.9 $\mu\text{g/mL}$, Level 2: 16.2 $\mu\text{g/mL}$, Level 3: 27.4 $\mu\text{g/mL}$. The range of analyte values that can be measured directly from the specimen without any dilution or pretreatment is 0.4–40.0 $\mu\text{g/mL}$. Samples with results in excess of 40.0 $\mu\text{g/mL}$ were repeated on dilution. The measured free PHT concentration in serum (dphF) was quantified from the probe after centrifugation for 20 min (at 1000–2000g) through a 30 kD cut-off filter to eliminate the protein-bound fraction. Free PHT from the filtered centrifugate was quantitated by HPLC (Agilent Technologies®). Calculation of the free PHT fraction dphCF from the dphT was performed using the Sheiner-Tozer formula (Fig. 1). This included: serum albumin concentration, total PHT serum concentration [dphT (mg/L)] and the theoretical unbound serum fraction (10 % for PHT) which corresponded with factor 0.1 in the algorithm [17]. In the formula denominator, the estimated bound fraction of PHT (90 %) is represented by factor 0.9. This bound fraction is multiplied by the normalized serum albumin value [individual albumin

concentration (g/L) divided by the mean serum albumin value of 44 g/L] and added to the free PHT fraction (10 %) of 0.1 in order to determine the values for free concentration in g/L.

The data was compared with the measured free PHT concentration and statistically assessed using non-parametric Spearman's rank correlation coefficient tests, t tests, Passing–Bablok regression analyses, as well as a Bland–Altman plot.

Results

Table 1 presents the PHT and albumin data of the 23 subjects. The following mean values and standard deviations (SD) were obtained: dphT = 7.5 mg/L, SD = 4.0 mg/L; dphF = 1.1 mg/L, SD = 0.5 mg/L; dphCF = 1.2 mg/L, SD = 0.7 mg/L. The mean albumin level in the patients ($n = 23$) was 25.5 g/L, SD = 4.7 g/L. In the low albumin group ($35 \leq \text{albumin} \leq 25$ g/L), the mean difference between dphF and dphCF was 0.10 (SD = 1.4). In the very low albumin group (< 25 g/L), the mean difference was 0.13 (SD = 0.24). An independent sample t test revealed no significant discrepancy in the mean differences between dphF and dphCF in the two groups $t(21) = -0.28$, $p = 0.78$.

The prediction for the calculated free PHT (dphCF) correlated highly with the measured free PHT (dphF); the Spearman's rank correlation coefficient showed a value of $r = 0.907$, $p = 0.00$. A linear regression analysis based on the Passing and Bablok [18] procedure further revealed that the calculated free PHT (dphF) and the measured free PHT (dphCF) were nearly identical (Fig. 2). Both hypotheses $\beta = 1$ (slope value of 1 was enclosed in the 95 % confidence interval) and $\alpha = 0$ (the 95 % confidence interval for the intercept contained the value 0) were accepted. A Bland–Altman plot (Fig. 3) further supported that there were no systematic differences between dphCF and dphF, and a regression analysis showed no significant relationship in the discrepancies between the measurements and the true value (proportional bias). The regression coefficient of the difference between the methods on the average of the two methods was $\beta = -0.35$, $p = 0.10$.

Discussion

The results support and validate the use of the Sheiner-Tozer formula to calculate free PHT concentrations in the hospital setting, confirming its usefulness in the absence of a free PHT determination. The data presented in this study are comparable to a similar investigation by Dager et al. [19], which reported a relative difference of 12.4 %

Table 1 Total PHT levels [dphT] and measured free PHT fractions [dphF] in the investigated 23 subjects

Nr.	Total PHT level (mg/L) [dphT]	Measured free PHT fraction (mg/L) [dphF]	Calculated free PHT fraction (mg/L) [dphCF]	Albumin (g/L)
1	2.58	0.30	0.38	28
2	3.20	0.35	0.49	27
3	3.83	0.38	0.54	30
4	2.88	0.51	0.56	20
5	4.75	0.57	0.67	30
6	2.50	0.59	0.44	23
7	2.83	0.60	0.55	20
8	4.78	0.67	0.78	25
9	8.73	0.91	1.10	34
10	3.68	0.96	0.75	19
11	11.03	0.97	1.39	34
12	8.35	0.98	1.28	27
13	5.08	0.98	0.92	22
14	9.80	1.24	1.66	24
15	6.70	1.37	0.94	30
16	10.38	1.53	1.76	24
17	10.58	1.64	2.00	21
18	11.05	1.70	2.09	21
19	16.15	1.78	2.33	29
20	10.68	1.78	1.87	23
21	9.95	1.89	1.32	32
22	9.30	1.93	1.90	19
23	14.40	1.93	2.44	24
Mean	7.5	1.1	1.2	25.5
SD	4.0	0.5	0.7	4.7

Albumin < 25 (n = 12)

Albumin ≥ 25 (n = 11)

t Statistic

Independent samples t test of the mean difference between dphF and dphCF for patients with: albumin < 25 (n = 12) compared to albumin ≥ 25 (n = 11)

Mean difference = 0.13
(SD = 0.24)

Mean difference = 0.10
(SD = 1.4)

t(21) = -0.28, p = 0.78.

The calculated free PHT level [dphCF] was determined according to the Sheiner-Tozer algorithm. SD Standard deviation

between measured and calculated values. The characteristics of the two studies are also similar: 23 patients in this study and 29 in Dager's, and both include adults with hypoalbuminaemia levels of <35 and <25 g/L, respectively. The patient subgroups in this study with <25 g/L albumin showed no significant deviation differences when compared to the whole group. Hong et al. [20] presented a much higher mean difference between measured and calculated free PHT of 0.65 mg/L (SD = 0.88 mg/L), which compromised the accuracy and the usefulness of the results. However, our study population was different to those in the Hong et al. investigation, as ours were European/Caucasian instead of Asian. This can influence the metabolic pattern for PHT significantly due to the different

genetic effects on its drug metabolism as well as on the free versus total PHT concentration and their kinetics. In their study, Wolf et al. [21] determined a difference between calculated and measured free PHT concentration of 0.31 mg/L (SD = 0.5 mg/L), which is also much higher than the results of this study. However, they reported solely on critically ill pediatric patients whereas we had only one pediatric patient. We looked at a much broader range of patients; not only with respect to age, but also severity of disease, and individuals outside the ICU. The data variability in our patient group, also indicated by the relatively high SD may explain why one (Bland–Altman plot) and two (Passing–Bablok regression) of the values were outside of the 95 % confidence interval, respectively. The values,

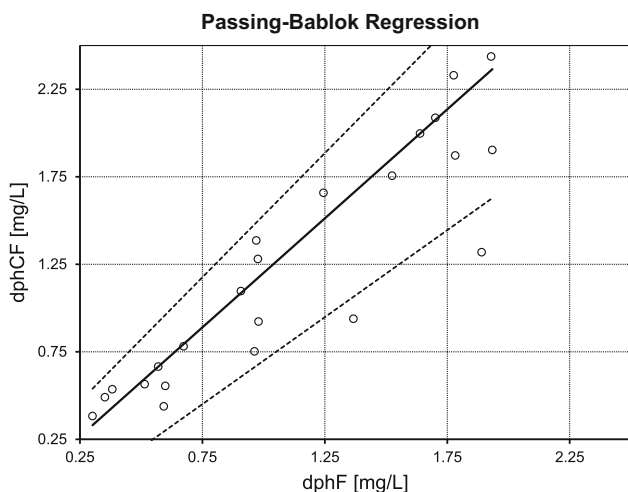


Fig. 2 Results of the linear regression analysis ($n = 23$) based on the Passing and Bablok [18] method, which revealed that the calculated free PHT (dphCF) and the measured free PHT (dphF) are comparable. Both hypotheses $\beta = 1$ (the slope value of 1 was enclosed in the 95 % confidence interval) and $\alpha = 0$ (the 95 % confidence interval for the intercept contained the value of 0) were accepted. Slope $\beta = 1.245$, 95 % CI [0.994, 1.419]. Intercept $\alpha = -0.044$, 95 % CI [-0.295, 0.112]. The *solid line* represents the linear regression. The *dotted lines* show the 95 % confidence interval

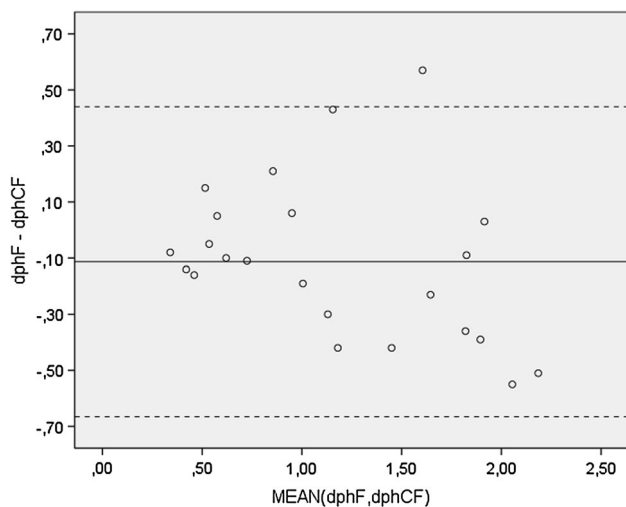


Fig. 3 Bland–Altman plot of the dphF and dphCF measurements ($n = 23$). The mean difference is $M = -0.11$ ($SD = 0.28$), which is not significantly different from zero ($t(22) = 0.07$). The *solid line* represents the mean difference. The *dotted lines* show the limits of agreement (representing ± 1.96 SD). In addition, regression analysis showed a non-significant relationship in the difference between the measured and the true values (proportional bias). The regression coefficient of the difference between the methods on the average of the two methods was $\beta = -0.35$, $p = 0.10$

however, were nearly very close to the limits of agreement of the statistical analysis. Accordingly the Sheiner-Tozer equation was helpful in estimating the free concentration

needed for careful dose adaptation—even in an unselected patient group. Continuing investigations with a more highly defined patient group could further validate the equation for its timely and correct use in clinical settings; especially when free drug concentrations are not available to guide optimal dosing of such a critical dose drug.

The importance of albumin and the related binding capacity in strongly protein bound drugs like PHT is controversially discussed in the context of a PHT TDM. According to Hong et al., hypo-albuminaemia not only modifies the ratio of free/total PHT concentration, but also the total PHT concentration, yielding incorrectly calculated results. This would also explain their data, at least for the pediatric patients [20]. They suggest determining free PHT in hypoalbuminemic patients using a suitable lab test, but such testing is often difficult to access or delivers data too late to be used for timely dose modification at bedside. One must also bear in mind that non-routine lab tests also result in higher administrative costs (availability, time and expenses). Therefore, such lab tests are often only ordered in specific situations when PHT dosing problems or a lack of drug effectiveness have occurred or is presumed. Our study demonstrates that in such cases, a lacking free lab PHT concentration can be calculated using the Sheiner-Tozer formula which was useful in a general hospital population, including patients with other potentially interacting medical treatments and a variety of diseases.

According to Krasowski and Penrod [22], the Sheiner-Tozer equation for calculating free PHT concentrations more frequently underestimates than overestimates the measured free PHT relative to the respective therapeutic ranges. Nevertheless, they also conclude that if measured free PHT concentration is not available, PHT concentration—adjusted according to the Sheiner-Tozer equation can supplement total PHT concentration and assist proper dosing. This was illustrated in this study on patients with low plasma albumin, a common condition in acute care hospitals. Our results in an unselected patient group with partial and even severe hypo-albuminaemia showed the Sheiner-Tozer formula to be an useful tool which yields comparable data to specific lab testing for free PHT. This facilitates a good TDM and provides additional information for drug dosing. In partial contrast to the published data, the measured free PHT concentrations values in our (almost completely) adult patient group varied only minimally. In addition, we did not detect any higher variation, either in low or very low hypo-albuminaemia in this small study group, nor was there a trend or indication of overestimation or underestimation of PHT concentrations. As a consequence, we were able to validate the usefulness of calculating the dphCF according to Sheiner-Tozer and can support its use in absence of measured free PHT values for an easy to do and appropriate dose adaptation at almost no

costs of hospitalized patients, when a TDM including free PHT determination is missing.

Finally, such a tool is also helpful to avoid medication errors in individualized patient treatment when checking a critical PHT dosing, or even in cases of poly medication with potentially interacting drugs, e.g. in relation to albumin binding despite a specific variable for interacting drugs in the equation.

Although the subjects came from different wards and medical disciplines, a limitation of our study is the relatively small number of patients and the low number of infants and younger children. Therefore, a comparison of different types of subjects, for example from ICU, surgical or internal medicine patients was not possible. To further elucidate our findings and add necessary details, a prospective study would assist in expanding and strengthening the evidence demonstrated to optimize TDM and the use of the Sheiner-Tozer equation for critical dose medications. It could then also validate the cost-effectiveness of the tool to avoid medication errors for better PHT management in hospitalized patients, as also indicated by von Winckelmann et al. [23]. Such medication support could be passed on to a multidisciplinary team and involve the pharmacist taking responsibility for an appropriate TDM service or to support the drugs and therapeutics committee by implementing therapeutic guidelines. Despite potential pharmacological interactions (most of the patients were receiving multiple drug therapy in ICU) we were not able to identify a distinct subgroup due to the small patient number. The data presented only minor discrepancies and were too variable to elucidate significant differences [see also the Passing–Bablok regression or the Bland–Altman plot where 2/23 and 1/23 patients fell outside of the 95 % limit, respectively (Figs. 2, 3)]. Nevertheless, the investigation indicated the potential benefit of the Sheiner-Tozer equation and suggests its use when a specific TDM for free PHT is not available. It provides a simple method to estimate free drug concentration in the clinical setting. Therefore and to further confirm our findings and add necessary details, an appropriately powered prospective study, could expand and strengthen the evidence demonstrated to optimize TDM and its usefulness for critical dose medications in such patients where there is a need to know the free drug concentration in hypo-albuminaemia.

Conclusion

Calculation of free PHT concentrations using the Sheiner-Tozer formula is a useful method to obtain additional information from total PHT concentration values and the albumin concentration in hypoalbuminemic patients in the absence of a specific, potentially time-consuming and

costly free PHT determination. This study on a small group of patients could not detect any specific difference in predicting the free PHT fraction between hospitalized patients from different wards, and from those with differing medical conditions.

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**A Quantitative Phenytoin GC-MS Method
and its Validation for Samples
from Human ex situ Brain Microdialysis,
Blood and Saliva Using Solid-Phase Extraction**

Andrea Tobler, Raphael Hösli, Stefan König & Stefan Mühlebach

A Quantitative Phenytoin GC–MS Method and its Validation for Samples from Human *ex situ* Brain Microdialysis, Blood and Saliva Using Solid-Phase Extraction

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This study describes the development and validation of a gas chromatography–mass spectrometry (GC–MS) method to identify and quantitate phenytoin in brain microdialysate, saliva and blood from human samples.

A solid-phase extraction (SPE) was performed with a nonpolar C8–SCX column. The eluate was evaporated with nitrogen (50°C) and derivatized with trimethylsulfonium hydroxide before GC–MS analysis. As the internal standard, 5-(*p*-methylphenyl)-5-phenylhydantoin was used. The MS was run in scan mode and the identification was made with three ion fragment masses. All peaks were identified with MassLib. Spiked phenytoin samples showed recovery after SPE of ≥94%. The calibration curve (phenytoin 50 to 1,200 ng/mL, $n = 6$, at six concentration levels) showed good linearity and correlation ($r^2 > 0.998$). The limit of detection was 15 ng/mL; the limit of quantification was 50 ng/mL. Dried extracted samples were stable within a 15% deviation range for ≥4 weeks at room temperature. The method met International Organization for Standardization standards and was able to detect and quantify phenytoin in different biological matrices and patient samples. The GC–MS method with SPE is specific, sensitive, robust and well reproducible, and is therefore an appropriate candidate for the pharmacokinetic assessment of phenytoin concentrations in different human biological samples.

Introduction

Epilepsy is a disorder of the central neural system characterized by recurrent unprovoked seizures caused by excessive discharge of electrical activity (1). Epilepsy can be treated with different antiepileptic drugs that generally render 80% of newly diagnosed patients seizure-free (2). Phenytoin (PHT) [C₁₅H₁₂N₂O₂ (3); molecular weight: 252.3 (3)] is a well-established antiepileptic drug designed to prevent and treat seizures (4). It is routinely used on neurosurgical intensive care unit patients with brain injuries.

PHT has a small therapeutic index; its therapeutic concentration range in blood serum is 10–20 mg/L (40–80 μmol/L) (5) for adults and children older than 3 months. Ingestion of more than 20 mg/kg in humans [normal oral dosage for adults is 6 mg/kg (5)] usually results in clinical toxicity (6). PHT can produce significant dose-related toxicity because of its complex pharmacokinetics (PK). The limited therapeutic index, combined with the large inter-individual variability of metabolism [half life 16–60 h, depending on plasma levels (7, 8)], and the nonlinear pharmacokinetics of PHT (9, 10) highlight the importance of therapeutic drug monitoring (TDM). On the other hand, relatively few studies have been able to demonstrate the benefits of TDM of antiepileptic drugs

(11). However, therapeutic monitoring of PHT using Bayesian forecasting was successfully applied to rapidly achieve therapeutic plasma levels using an easy-to-apply PHT loading dose regimen in a hospital setting (12).

The monitoring of brain tissue biochemistry during intensive care cerebral microdialysis is well established (13). Samples generated by brain microdialysis have the potential to correlate PHT concentrations at the site of action, with plasma values mostly used for TDM (14, 15).

The correlation of toxicity and plasma level concentration is well established. However, the PK in critically ill patients, and its potential change related to the characteristics of the blood-brain barrier, contribute to the lack of understanding of the kinetics and mode of action of antiepileptics in the brain. The correlation between PK in blood and the target tissue in brain is not established. To investigate correlations of PHT concentration in blood plasma, saliva (oral fluid) and tissue microdialysate, a sensitive and specific analytical method is needed (16, 17). Saliva has been shown to serve as an alternative sample to blood plasma for TDM (18).

Commonly used analytical methods without mass spectrometry (MS) detection do not reach the sensitivity needed to quantify the free fraction of PHT and the low biological PHT concentrations (19). For clinical trials, a validated method is required by authorities such as the Food and Drug Administration (FDA). Gas chromatography (GC) with MS is effective and specific to separately analyze parent PHT and its metabolites (20). However, current published GC–MS methods are not accurate and sensitive enough to identify and quantify the expected low levels of PHT in microdialysate samples from the human brain. Also, the high sample volume of 500 μL used in a recently published study is far too large for the usual microdialysis sampling in patients, with a 2 μL/min flow rate, and therefore not appropriate for TDM (21). GC–MS analysis needs an extraction step such as solid-phase extraction (SPE) to clean the sample and to eliminate interfering biological matrix materials like proteins and lipids prior to injection into the GC–MS system. Furthermore, to detect PHT with sufficient sensitivity by GC–MS, a derivatization of PHT is necessary (22, 23). A structural analogue to PHT (C₁₆H₁₄N₂O₂; molecular weight: 266.3), 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH), is chosen as an internal standard (IS) (24).

The aim of the present analytical study was to establish a selective and sensitive GC–MS method allowing the determination of PHT in different human biological samples, especially in brain microdialysates. The analytical method should cover a therapeutic range of free PHT concentration ranging from 50 to 1,200 ng/mL. A further objective of this study was to develop a

simple and effective sample extraction method, which can be used for different biological matrices like blood, dialysate or saliva to reproducibly provide stable, reliable and clean analytes for GC–MS analysis. The suitability of the analytical GC–MS method has to be demonstrated by validation according to International Organization for Standardization (ISO) 17025 to be used in corresponding investigations with samples from patients (clinical trials). The resulting analytical method is a prerequisite for further PK and pharmacodynamic (PD) investigations.

Material and Methods

Chemicals and samples

The PHT reference was purchased from Desitin Pharma GmbH (Liestal, Switzerland) and from the European Pharmacopoeia (Strasbourg, France). The IS, MPPH ($C_{16}H_{14}N_2O_2$; molecular weight = 266.29), purity >99% was purchased from Sigma-Aldrich Chemie (Steinheim, Germany). Calcium chloride, citric acid monohydrate, potassium chloride, magnesium chloride hexahydrate, sodium chloride and sodium hydroxide and the solvents methanol (MeOH), acetic acid (100%) and acetone were of analytical grade and purchased from Merck (Darmstadt, Germany). Trimethylsulfonium hydroxide (TMSH), 0.2M in MeOH for derivatization, was purchased from Macherey-Nagel (Oensingen, Switzerland). Nitrogen (analytical grade) for extract drying, and helium (analytical grade) for GC, were obtained from Carbagas (Liebefeld, Switzerland).

Artificial cerebrospinal fluid (aCSF), composed of 8.59 g NaCl, 0.2013 g KCl, 0.1332 g $CaCl_2$ and 0.1758 g $MgCl_2 \times 6(H_2O)$, was prepared according to the pre-clinical device company CMA (Stockholm, Sweden; dialysate solution) (25). CPDA-1 blood (an anticoagulant citrate phosphate dextrose adenine solution; subsequently referred to as blood plasma) was obtained from the Blood Donor Center in Bern, Switzerland. Saliva was provided by a volunteer. *Ex situ* brain tumor tissue, which had been surgically removed from patients treated with PHT, was dialyzed against aCSF with a flow rate of 2 μ L min, yielding a sample of approximately 60 μ L. Approximately 2 mL of patient CPDA-1 blood samples with PHT were collected. Both samples (dialysates and CPDA-1 blood) were provided by the Department of Neurosurgery (Kantonsspital Aarau AG, Switzerland). The sampling procedure was presented to the ethical committee of the Kantonsspital Aarau, which did not require formal ethical approval because the dialysates were made *ex situ*. All biological samples (CPDA-1 blood plasma and dialysate) were frozen immediately after sampling at $-24^\circ C$. For transportation, a refrigeration chain ($-20 \pm 2^\circ C$) was guaranteed. Before extraction and analysis, the samples were thawed at room temperature for 30 min and then vortexed for 1 min.

IS, calibrator standard solutions, quality controls, system suitability test and sample preparation

MPPH (IS) 1 mg/mL in MeOH was prepared as a stock solution and stored at $2-8^\circ C$. This stock solution was diluted with MeOH to 100 ng/ μ L for addition to solutions [internal standard solution (ISS), 100 ng/ μ L]. The PHT reference stock solution containing 1 mg PHT/mL MeOH was stored at $2-8^\circ C$. To obtain

calibration standard solutions (Cals) of 1,200 (Cal 6), 1,000 (Cal 5), 800 (Cal 4), 300 (Cal 3), 150 (Cal 2) and 50 ng/mL (Cal 1), the stock solution was diluted freshly each time with MeOH for each measurement. To each calibrator standard (Cal), 1,200 ng/mL MPPH (12 μ L ISS) was added as IS. To each Cal, 1 mL of biological matrix (aCSF, blood plasma or saliva) was added for validation purposes. For quality control (QC), solutions were prepared with 100 ng/mL (QC 1) and 1,000 ng/mL (QC 2) PHT, also containing 1,200 ng/mL MPPH (12 μ L ISS) as IS and 1 mL of blood plasma, aCSF or saliva matrix. The system suitability test (SST) was prepared with the PHT reference solution and IS, to a final probe of 100 ng/mL PHT and 1,200 ng/mL MPPH (12 μ L ISS).

Samples from patients were included to test the method in real biological probes. PHT-containing microdialysis samples (volume of 50 μ L) from six patients (requiring approximately 25 min of collection time) were added to 450 μ L aCSF and 1,200 ng MPPH (12 μ L ISS) as IS prior to extraction. Blood plasma samples (volume of 500 μ L) from PHT-treated patients were spiked with 1,200 ng MPPH (12 μ L ISS) as IS. The volumes of dialysate and blood were not identical to the volumes used in the validation procedure; however, they were useful for comparing artificial and real samples.

SPE procedure

For SPE, cartridges were used with nonpolar C8 sorbent and a strong cation exchanger (SCX) (Bond Elute LRC Certify, 130MG; Varian). The extraction procedure is shown in Figure 1. The method was adapted from literature (26). After equilibration of the SPE columns with 1 mL acetonitrile for approximately 2 min, the pH was adjusted with 1 mL citric buffer, pH 5.0 (20.1 g/L citric acid, 8.0 g/L NaOH plus pH adjustment with 1 M HCl) (27). The calibrators (1 mL), QCs (1 mL) and samples (0.5 mL) were applied to the SPE columns (Figure 1). The SPE columns were washed with 1 mL citric buffer, followed by 1 mL of 0.01 M acetic acid for pH adjustment (pH \sim 3.5). After application of a vacuum (approximately 0.5 bar) and drying for 5 min (using a vacuum pump; Vac Master KNF LAB Laboport), PHT was eluted with 2×1 mL acetone. The columns were vacuum-dried again for 1 min at 0.5 bar. The eluate was transferred into a 2 mL vial and evaporated with nitrogen at $50^\circ C$. The reconstitution and derivatization were performed with 50 μ L TMSH immediately before GC–MS analysis at room temperature and vortexed for 10 s (22, 28, 29).

GC–MS analysis

To demonstrate the robustness of the developed method, two different GC–MS systems available in the same lab were used. An HP5890 gas chromatograph was used, connected to an HP5971 mass selective detector (with Chemstation software supplied by Hewlett Packard). The backup system was an Agilent 6890N/5973 Inert GC–MS. An autosampler and an injector were connected to both systems (Agilent 7673 with an injection syringe of 10 μ L volume). Two microliters of the prepared samples were injected to the liner prior to the GC column. The GC column was a polysiloxane Agilent J&W Capillary 122-5532, DB-5MS, length, 30 m; i.d., 0.250 mm; film, 0.25 μ m; for temperatures from -60 to $350^\circ C$. The temperature

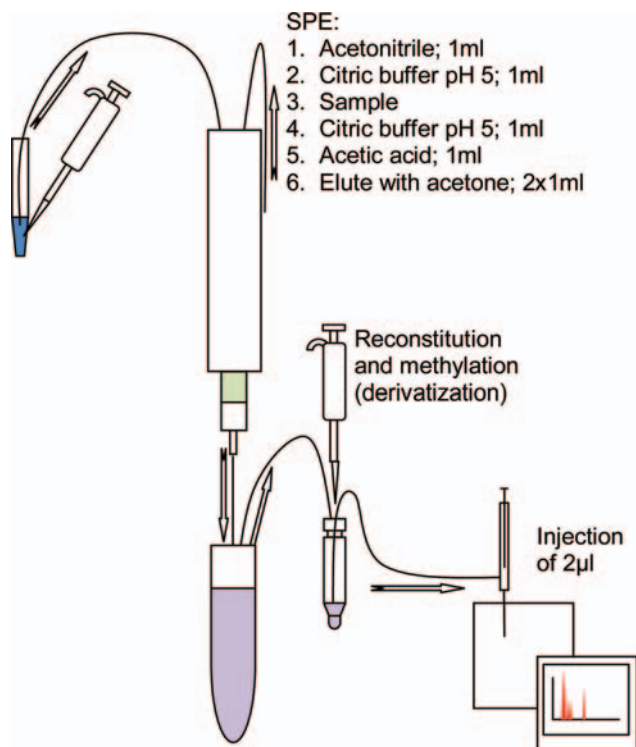


Figure 1. Schematic sample process: generation of the three sample matrices, processing during SPE and derivatization prior to GC–MS measurement.

program was set to 120°C for 1 min, then raised by 10°C/min to 300°C and held for 6 min. The carrier gas was 99.95% high purity helium with a flow of 1.6 mL per min. The MS systems were on scan mode to additionally check for interfering materials. The temperature of the MS detector was 150°C. The settings for the ion source and the transfer line were 230 and 280°C, respectively. The scan range was between 50 and 650 amu. The reference window for the data analysis parameters for PHT was 2 min, with a non-reference window of 1 min. All chromatographic peaks were analyzed with MassLib (www.masslib.com), allowing mass spectra identification by both similarity and identity. The search algorithm Search for Similar and Identical Compounds (SISCOM) originates from Henneberg, Weimann, and Ziegler (Max-Planck-Institute, Mühlheim a.d. Ruhr, Germany) and was developed in the 1970s (30).

Validation procedure

The validation according to ISO 17025 includes the assessment of selectivity, accuracy, recovery of PHT after SPE, reproducibility, suitability of the calibration curves, stability of PHT and matrix effects. Indicators for the sensitivity of the method were the accurate assignment of the ions in different matrices and the limit of detection (LOD) and limit of quantification (LOQ). Blank samples from six different sources were used to demonstrate selectivity.

Furthermore, the selectivity of the method was determined by using the selected GC column combined with an MS detector. The selectivity was verified based on the retention time and the allocation of one quantifier ion and two qualifier ions. The

accuracy was tested at the same levels as the calibrators and with two additional levels at QC 1 (100 ng/mL) and QC 2 (1,000 ng/mL) ($n = 6$). Two different solutions (prepared from different stock solutions) were used to prepare the calibration and QC samples. The recovery of PHT was analyzed by measurement of QC 1 (100 ng/mL) and QC 2 (1,000 ng/mL) ($n = 3$), with QC 1 (100 ng/mL) and QC 2 (1,000 ng/mL) ($n = 3$) without SPE. The reproducibility and suitability of calibration curves was measured by 2×3 complete series of Cal 1 to Cal 6 samples, including the extraction step. Inter-assay percent relative standard deviation (RSD), linearity and regression coefficient were calculated. PHT stability was analyzed with QC 1 samples after extraction for both non-derivatized [5 weeks at room temperature ($n = 10$)] and derivatized samples (33 h without specific cooling). Matrix effects were analyzed by comparing the calibration curves generated with the three matrices: aCSF ($n = 6$ series), blood ($n = 3$ series) and saliva ($n = 3$ series). Thus, the values of each calibrator and matrix were also compared.

Statistical methods

The statistical data were calculated with Microsoft Excel and PASW Statistics 18.0. To show the similarity between the different matrices, a one-way analysis of variance (ANOVA) was used. Furthermore, for each matrix, the six calibrator levels were checked with t -tests to determine whether the measured values differed significantly within each level.

Results and Discussion

GC–MS analysis of PHT (separation and identification of PHT and MPPH), retention time, ion fragment mass, LOD and LOQ

PHT and MPPH have different, but comparable, retention times consistent with their chemical characteristics. The GC-MS chromatogram of PHT showed a retention time (RT) of 15.12 min and the IS has an RT of 16.15 min. The relative RT was 1:1.015. The suitability of MPPH as IS for GC–MS analysis of PHT was further supported by the chromatogram (Figure 2). The observed molecular fragment masses were [280, 203, 194, 118] for PHT and [294, 203, 194, 118] for MPPH. These minimal differences in the RT, and the comparable molecular fragment mass spectra, illustrate the strong similarity between the analyte PHT and the IS, MPPH. The calculation of the sample concentration was made only with the ion fragment masses that were identified for PHT and MPPH.

The MassLib system could easily detect PHT and MPPH separately by their mass chromatogram differences. Because of the independence of the data type and the format of the analyte, MassLib has been successfully used in earlier GC–MS studies, and is now widely used as a standard to identify chemical substances in toxicology (31). Also, these results were in line with prior studies and confirmed the suitability of this tool for the GC–MS analytical method to identify and quantify PHT.

The LOD was calculated as signal to blank noise ratio (S/N) ($> 3:1$). The LOD for this method in aCSF, saliva and blood was 15 ng/mL, according to the FDA guidelines or Deutsches Institut für Normung (DIN) standards (32, 33). Following the

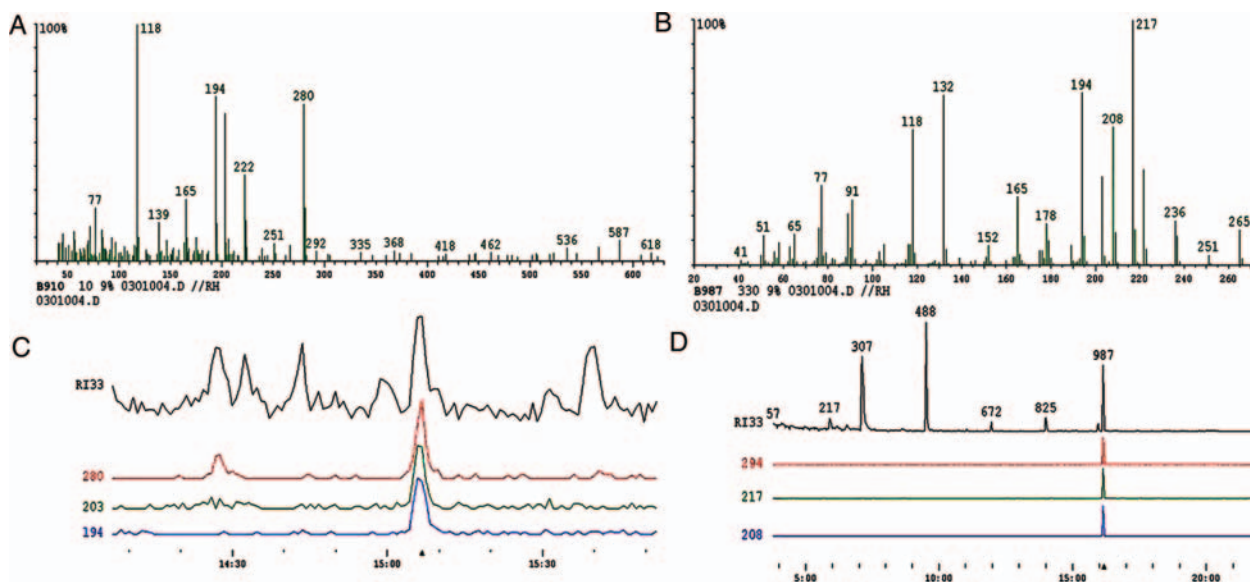


Figure 2. Total ion chromatograms (TICs) and MS fragment spectra of PHT (50 ng/mL) and MPPH (1,200 ng/mL). GC–MS chromatograms: (A) PHT; (B) IS MPPH; (C) MS fragments and a 2.0 mL injection of a 50 ng/mL PHT solution LOQ; (D) time-related peaks and 1,200 ng/mL MPPH. (A) The ion fragments for PHT are [280,203,194,118], (C) RI33: TIC chromatogram; chromatograms of the mass fragments at m/z 280, 203 and 194 and (B) for MPPH the fragments are [294,203,194,118], (D) RI33: TIC chromatogram; chromatograms of the mass fragments at m/z 294, 217 and 208.

FDA guidelines, the LOQ was 50 ng/mL PHT and calculated as five times the response/blank noise. Thus, the LOQ was reached at Cal 1 level (50 ng/mL PHT). This also corresponds to the FDA guidelines, claiming 20% reproducibility at the LOQ level (32, 33), which was reached with Cal 1.

Accuracy of the calibrators, repeatability precision and laboratory precision

For assessment of the accuracy, the calibrators and QCs of six individual measurements ($n=6$) were performed. The accuracy was tested with two non-identical solutions, the stock solutions for calibration and the one for QC samples. The results of the one-way ANOVA showed no significant differences for all matrices ($F=0.0002$; $p=0.9998$). In the target range of therapeutic PHT concentrations in microdialysates (>50 ng/mL, $<1,200$ ng/mL), accuracy was between 104.6 and 98.9%. As expected, the largest deviation was observed at the LOQ and at the lowest concentration level (Cal 1 at 50 ng/mL) of the calibration curve, showing an accuracy value of 104.6% (Table I). The calibrator values showed minimum and maximum percent deviations of 20% in Cal 1, 7% in Cal 2, 3% in Cal 3, 1% in Cal 4, 9% in Cal 5, 6% in Cal 6, 12% in QC1 and 6% in QC2 (Table I). Statistically, the deviations of each concentration value did not differ for the entire calibration, demonstrating that there were no outliers. All of the values were within the maximum allowed bias of 15% (32). Repeatability precision (inter-assay variability), the minimum and maximum deviation of all levels of measurements, was within 15% of the coefficient of variation (CV) (Table I).

Regarding laboratory precision (intra-assay variability), for the QC 1 and QC 2 measurements ($n=6$), the mean percent deviation from the target value was 2.7% for QC 1 (100 ng/mL) and 0.6% for QC 2 (1,000 ng/mL). As with the calibrators, the

QC 1 and QC 2 also showed a deviation from the nominal value of less than 15% CV. These results support the robustness of the method. Neither the operator nor the day of preparation influenced the results in a detectable manner. There was no difference in the characteristics of the chromatogram when changing from the GC–MS HP system to the backup (Agilent Systems). The laboratory in which the investigations were made is part of the forensic laboratories in Switzerland and takes part in all necessary proficiency tests to be ISO 17025 certified.

Linearity and regression coefficient of calibration curve

The linearity of the calibration was tested with replicates of Cal samples for the PHT concentration range of 50 to 1,200 ng/mL in aCSF ($n=6$), saliva ($n=3$) and blood ($n=3$). As shown in Table I, the calibration curve for PHT showed a linear regression coefficient (r^2) greater than 0.998 ($r^2_{\text{blood plasma}}=0.998$, $r^2_{\text{dialysate}}=0.999$ and $r^2_{\text{saliva}}=0.999$) in all three matrices, indicating excellent linearity in the target concentrations (Table I). The calculations, including the linearity check of the calibration curves, were directly conducted by the ChemStation software.

The one-way ANOVA showed no statistically significant differences in calibrator-spiked samples of the different matrices: $F=0.0002$, $p=0.999$. Each calibrator level was tested for consistency using a t -test. The t -test showed no statistically significant deviation at each calibration level. This allows for the validation procedure to be simplified by analyzing only one matrix and extrapolating the results to the other matrices. It is standard that the deviation of the lowest Cal from the nominal value should be within $\pm 20\%$. For all other Cals, the deviation from the nominal value should be within $\pm 15\%$, as required by FDA

Table 1

Calculated Data from the Measurements with aCSF as Matrix and Arithmetic Mean of the Calibrators in Different Matrices*

Calculated data from the measurements with aCSF as matrix

Sample name	Overall mean	SD	Mean deviation (%)	Inter-assay CV (%)	Accuracy (%)
Cal 1 (50 ng/mL)	52.3	6	9.65	11.5	104.6
Cal 2 (150 ng/mL)	152.5	16.9	7.51	11.1	101.7
Cal 3 (300 ng/mL)	296.7	33.3	9.33	11.2	98.9
Cal 4 (600 ng/mL)	597	55	6.75	9.2	99.5
Cal 5 (1,000 ng/mL)	996	48	3.55	4.8	99.6
Cal 6 (1,200 ng/mL)	1,220	65	4.46	5.3	101.7
QC 1 (100 ng/mL)	102.7	6	2.7	6.3	102.7
QC 2 (1,000 ng/mL)	1,005.7	35	0.6	3.6	100.6
Arithmetic mean of the calibrators in different matrices					
Calibrator (concentration)	aCSF mean/deviation from target concentration (<i>n</i> = 6)	Saliva mean/deviation from target concentration (<i>n</i> = 3)	Blood plasma mean deviation from target concentration (<i>n</i> = 3)		
Cal 1 (50 ng)	52.3/20%	42.8/14.4%	42/16.0%		
Cal 2 (150 ng)	152.5/7%	153/05.7%	154.6/4.8%		
Cal 3 (300 ng)	296.7/3%	287.8/6%	296.8/3.9%		
Cal 4 (600 ng)	597/1%	612 /1.9%	627/4.5%		
Cal 5 (1,000 ng)	996/9%	998/1.7%	972/6.67%		
Cal 6 (1,200 ng)	1,220/6%	1,206/1.1%	1,194/0.7%		
Linear best fit	aCSF	Saliva	Blood plasma		
Coefficient of determination (<i>r</i> ²)	0.999	0.999	0.998		
<i>y</i> -intercept	0.288	3.818	5.542		
Slope (<i>x</i>) calibration	1.006	1.002	0.989		

*Note: All samples were spiked with PHT. Mean deviation (%): mean percent deviation from target, inter-assay; CV (%): inter-assay percent RSD; accuracy (%): accuracy calculated out of each sample group in percent (*n* = 6 for all samples).

guidelines (32). The presented method fulfilled these requirements and even performed superiorly.

SPE; recovery with and without SPE

To produce reproducible GC–MS data from biological samples, an appropriate clean-up procedure of the samples is necessary to eliminate the matrix components that may potentially interfere with the analysis, to maintain the responsiveness, and to keep the method consistent and reliable, even after larger series of sample analyses. SPE is widely used for the extraction procedure in GC–MS analyses. SPE columns are commercially available, which allows high-grade and reproducible quality characteristics. The mixed-mode silica SPE columns used in this investigation are also frequently used in forensic toxicology to extract basic and cationic drugs, and are also very effective for the extraction of a wide range of compounds from aqueous matrices like urine, dialysate or blood (34). They are appropriate for small sample volumes and low concentrations of the investigated substances (28, 29), as was the objective in this study.

A single extraction required 40 min, including the time for admixing the solvents, equilibration, flow through the sorbent and drying of the eluate. This indicates that the method is not intended for routine serial TDM analyses, but fulfils the requirements for research.

The recovery was analyzed by the measurement of spiked QC 1 and QC 2 samples extracted in SPE columns, compared with spiked QC 1 and QC 2 samples without extraction. The difference between the PHT values of QC 1 and QC 2 with and without SPE was within an 8% range for QC 1 and 6% for QC 2, which demonstrates the efficiency and reliability of the SPE ($n = 3$). The inter-assay CVs (%) of the recovery were 15% for QC1 and 2% for QC2. The recovery of more than 90% of the spiked samples was consistent and reproducible ($n = 6$) and demonstrated that the SPE columns are suitable for the extraction of PHT from biological samples such as human blood plasma, saliva or brain microdialysate (32). The amount of PHT recovered after SPE was 94.1% for 100 ng/mL and 94.3% for 1,000 ng/mL, compared to the amount found in unextracted QC samples (100%) ($n = 3$).

Stability of the extracted PHT samples

The stability of the processed samples was tested over time by preparing and extracting QC 1 10 times. The eluate was evaporated (Figure 1) and the dried QC 1 samples were stored at room temperature. These samples were derivatized with TMSH at Weeks 0, 2, 3, 4, and 5 and measured immediately after derivatization. The variations of the measured concentration over time were within 15%. No significant changes, deviation or trend for degradation were detectable in the GC vials during storage. This leads to the conclusion that the dried PHT eluate was highly stable at room temperature and no time-dependent degradation occurred during the 5 weeks. In practical terms, these results demonstrate that the processed PHT samples (cleaned up) are stable and can be stored at room temperature without loss of PHT before TMSH derivatization for the GC–MS analyses.

The short time stability testing of derivatized QC samples, stored without artificial cooling at ambient temperature of approximately 30°C, performed three times at 0, 23 and 33 h, showed an even smaller deviation than those in the underivatized study over 5 weeks. The deviations from the first measurement were within 4% at 100 ng/mL and 1% at 1,000 ng/mL. There was also no statistically significant decrease in the concentrations of PHT or IS over time, demonstrating the stability of the derivatized samples over at least 24 h at slightly elevated temperatures (e.g., in a probe tray or autosampler), even when the vial cap was perforated by the injection needle. The analysis of the underivatized stored samples after SPE showed at 100 ng/mL a deviation from the first measurement within 3%, and at 1,000 ng/mL a deviation of 5%. The stability data from the derivatized and non-derivatized groups that were simultaneously assessed showed no significant variations between the groups. Therefore, biological PHT probes (after SPE and evaporation) are very stable at room temperature over more than a month, and are not affected by conditions encountered in an ordinary analytical lab. No specific storage precautions are needed.

Selectivity and specificity

The selectivity and specificity were demonstrated in all three matrices (blood plasma, dialysate and saliva) by good peak differentiation and quantification of PHT. Both the blank biological samples without IS and those with IS were negative; hence, any false positive blank samples could be excluded (aCSF: $n = 6$; saliva: $n = 3$; blood plasma: $n = 3$).

Samples from patients receiving PHT

To test the method on real human biological samples, *ex situ* brain tumor microdialysates and blood samples from six patients treated with PHT were analyzed. The volumes of dialysate and blood were not identical to the sample amount in the validation (described previously). Nevertheless, the first indication on the use of the method was observed in non-spiked biological samples (patients' probes). The results of the dialyses samples were (in ng/mL) 54.8 for Patient 1, 162.6 for Patient 2, 45 for Patient 3, 63.4 for Patient 4, 353.2 for Patient 5 and 661 for Patient 6. The corresponding blood values (in ng/mL) were 2,245 for Patient 1, 3,078 for Patient 2, 5,676 for Patient 3, 19,073 for Patient 5 and 16,349 for Patient 6. No blood was available from Patient 4. The blood and dialysate data from the individual patients were consistent. Only 1–5% of blood plasma in brain *ex situ* dialysates resulted, which corresponds to approximately half of the assumed free serum PHT concentration (10% free PHT in plasma). The measured PHT concentrations were in the tested range of the method. It has to be speculated that in the monitored ICU patients, either steady-state conditions in the brain (deep compartment) were not yet achieved, or other specific PK conditions existed, indicating a more complicated extrapolation from plasma to the brain values (blood-brain-barrier or leaking central compartment). No saliva (oral fluid) samples were collected and analyzed from patients. The results show the importance of testing and validating a new TDM analysis. The need for parameter validation, including correct modeling of data, is crucial for reliable drug

concentration tracking in biological materials intended to investigate PK/PD correlations in such critical patients. Such studies could result in safer, more efficacious and rational drug dosing in patients.

Conclusions

The goal of the study was to establish a selective and sensitive GC–MS method that allows for the determination of PHT in different biological samples matrices such as blood plasma, saliva or brain microdialysate for TDM and related PK/PD investigations.

The robustness of the method was illustrated by using two different analytical systems, which did not show any differences in the response and results. There was no deviation between the measurements made for the calibration curve with different matrices, and furthermore, the QC of the measurements with different matrices also showed no differences. The presented method has been proven for PHT detection and quantification in the aimed investigated human biological matrices. This reveals the exemplary robustness of the method.

The cleaned samples are stable for at least one month at room temperature before derivatization for GC–MS. They were stable for more than 30 h in derivatized form, ready to be analyzed in an autosampler. The LOD (15 ng/mL) and the LOQ (50 ng/mL) meet the requirements of FDA guidelines and the method meets analytical standards according to ISO 17025. Therefore, the method is suitable for assessing PHT in different tissues, including liquor/brain microdialysate at therapeutic concentrations (100–200 ng/mL). The method might be of primary interest for research purposes such as toxicity, clinical trials or PK/PD investigations of patients in critical care with brain surgery/trauma in which microdialysis is used.

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6 CONCLUSIONS

Bayesian Forecasting (BF) versus conventional dosing (CD): a retrospective, long-term, single centre analysis

Correct PHT dosing and administration is difficult and prone for inappropriate or even incorrect procedures (medication errors) which are also characteristic for narrow therapeutic dose drugs. In hospitals, rapid IV loading of PHT as antiepileptic is often necessary but should be individualized and accompanied by a validated therapeutic guidance including TDM recommendations and proper support to interpret the data [1-6]. As shown in this long-term investigation, BF together with a strict and detailed PHT dosing/infusion protocol is by far superior to CD. The presented regimen includes an initial high (15mg/kg body weight) IV loading dose over a defined slow infusion period. It is well-tolerated and accompanied by a rational TDM and is suited for the clinical setting. With only the body weight of the patient, appropriate population data, and one to three non-steady-state serum level determinations during the loading phase an accurate BF of an individual PHT dosage is provided in almost all hospitalized patients. The initial standardized high dose PHT loading over five days was effective and safe. The TDM lab service together with the medication support service from the hospital pharmacy allowed a successful multidisciplinary implementation and follow-up of the therapeutic PHT guidelines. Continuous support, active teaching and accessibility of this dosing support are necessary because of the rapidly changing trainee staff in a teaching hospital as also demonstrated in this study. BF is powerful and efficient for drug dosing and TDM for both, the routine (chronic) treatment and also the extraordinary case (emergency, ICU).

Recommendations from the investigations

- BF patients showed tremendously improved therapeutic PHT serum levels compared to CD patients. This can only be achieved and maintained in a teaching hospital by a permanent instruction, follow-up and an interprofessional approach for the medication management [7]. Nevertheless, additional prospective investigations with outcome-measurements (e.g. morbidity like seizures, length of hospitalisation etc.) are needed to correlate the improved serum levels also with clinical outcome.

- The large data set from this long-term investigation could be used to generate new and even better (hospital focussed) population data. Such Michaelis-Menten parameters (V_d , v_{max} , K_m) from adults incremented by data from children would foster the proposed regimen (see below).
- Children (≤ 40 kg body weight) represented only a small group in this study, and should be studied in-depth to evaluate e.g. for a (lowered) maintenance dose in this sensitive group of patients.
- Special care has to be given for the interpretation of serum levels in the elderly to respect the altered pharmacokinetics like reduced albumin concentrations, diminished efficiency of drug metabolism (CYP-enzymes). In this cases, the Bayesian forecasting is superior, because it calculates further parameters changing with age such as V_d and K_m .
- The interprofessional collaboration between laboratory, hospital pharmacy, medical and nursing staff improves the efficiency and correctness of difficult to dose drugs such as PHT.
- A patient-centred but also pharmacological input especially in the absence of clinical pharmacology service on site can be timely provided from the appropriately trained hospital pharmacy team to support a rational and safe drug use in difficult drug therapies as also included in the overarching statements of the International Pharmaceutical Federation (FIP) to provide good pharmacy practice (GPP), good trade practice (GTP), good manufacturing practice (GMP) and good distribution practice (GDP) together with good procurement practice to the point of care in multiprofessional teams to improve treatment results and patient-benefit [8].
- TDM in the interdisciplinary collaboration between the clinics and the hospital pharmacy can efficiently support the rational and good drug use and has to be considered when using drugs like PHT with a narrow therapeutic range.

Free PHT assessment

Total PHT serum levels are well established and used. But they don't reflect the effective free drug concentration relevant at the site of action (brain). Calculation of free PHT concentrations using the Sheiner-Tozer formula is a useful, quick and an easy method to obtain necessary additional information from total PHT and the albumin concentration in hypoalbuminemic patients in the absence of a specific, potentially time-consuming and costly free PHT determination to avoid medication errors for better PHT management in patients [9]. This investigation in a small group of patients could not detect any specific difference in predicting calculated free PHT fraction between hospitalized patients from different wards, and from those with different medical conditions nor in relation to low or very low albumin levels compared to measured free PHT levels.

Recommendations

- The Sheiner-Tozer equation supports a calculation of free PHT concentration from only measured total PHT and the known albumin concentration in a simple and rapid way when a free PHT determination is lacking.

GC-MS Analysis of biological PHT samples

A selective and sensitive GC-MS method has been developed and validated that allows the determination of PHT in different biological sample matrices such as blood plasma, saliva or brain microdialysate for TDM and related PK/PD investigations. The LOD (15 ng/mL) and the LOQ (50 ng/mL) meet the requirements of the FDA guidelines or of the Deutsches Institut für Normung (DIN) standards. The method meets analytical standards according to ISO 17025 [10-11]. Therefore, the method is suitable for assessing therapeutic concentrations (100–200 ng/mL) of PHT in different tissues, including liquor/brain microdialysate sometimes available in neurosurgical service.

The sample preparation and clean-up using SPE was efficient, reproducible and showed an almost complete recovery of > 94%.

The analytical GC-MS method fulfilled the requirements for a selective, sensitive, linear, precise, stable, reproducible and robust analysis suitable for the determination of (free) PHT in the CNS-dialysate.

Further studies

- The defined analytical method and their validation represent as a base to further investigate PHT in (human) biological samples (blood, saliva,...) with primary scientific interest e.g. in the PK-PD area in patients.
- The method should be further evaluated with other analytical and detection systems like LC-MS to get a robust, simple and economic analysis also to simplify and e.g. to eliminate a clean-up step of the samples when using patient samples.
- The correlation of PHT concentration in the serum and the extracellular fluid will be further investigated also using kinetic modelling to assess the power of serum TDM also in specific patient groups (thesis Raphael Hösli).

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8 POSTER PRESENTATIONS

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10 CONGRESS PARTICIPATIONS

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