

# **Advances in Therapeutic Drug Monitoring with a Focus on Phenytoin Analysis**

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Dekan der Philosophisch-  
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Dedicated to my Family

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## ABSTRACT

With the growing demand for a therapeutic drug monitoring (TDM) in clinical laboratories the development of appropriate and improved analytical methods to monitor drug concentrations and optimize patient care has become imperative. This dissertation contributes in this regard by developing sensitive, specific, and reliable GC-MS and LC-MS/MS methods to measure drug concentrations and validating them according to the FDA Guidance for Industry. The methods are performed based on the example of the critical dose drug phenytoin (PHT), which has characteristics – such as a small therapeutic range, nonlinear pharmacokinetics, interactions with other drugs, inter-individual genetic differences in the metabolism, and long-term therapy – that render it a highly suitable model substance and a reliable standard for the development and application of analytical methods to measure concentrations also of other medications.

Both methods allow the measurement of low drug concentrations in different body compartments (i.e., blood, saliva, and samples from brain tissue microdialysis with relatively small sample volumes) and therefore could be used to measure drug concentrations in the compartment of interest for various other drugs. By systematically comparing the two methods, the dissertation concludes that although GC-MS is a sensitive and suitable method (e.g., for the measurement of volatile and thermally stable substitutes in a sample), the LC-MS/MS method offers significant advantages (in terms of its sensitivity, LOD/LOQ, needed sample volume, sample preparation, analysis time, and costs), which make it highly suitable for larger sample

numbers as in pharmacokinetic/pharmacodynamic studies as well as for bedside and routine analyses. While these findings are based on the model substance PHT, other promising drugs for TDM are, for example, Midazolam, Ceftriaxon, Morphine, and Levatiracitam, which are often administered concomitantly in neurosurgery. In the future, the routinely used matrices (i.e., blood or urine) for TDM will be expanded with other sample sources like tissue biopsies, dried blood spots, and oral fluids. LC-MS/MS methods provide the advantage of drug quantification in such matrices which are easier to handle. Thus, the dissertation concludes that LC-MS/MS methods are the current gold standard for TDM.

Furthermore, in this dissertation also a theoretical estimation method to calculate free drug concentrations has been tested, which could be used in the absence of corresponding lab methods or data – not every hospital or institution is able to afford a LC-MS/MS or a GC-MS instrument. The dissertation tested the usefulness of the Sheiner-Tozer algorithm for the correct estimation of the free PHT concentrations in hypoalbuminemic patients and compared the estimated with the measured free PHT levels. There were no statistically significant differences between the two methods and the estimated values highly correlated with the measured free PHT values. The results support the usefulness of the Sheiner-Tozer formula to calculate free PHT concentrations from the total PHT and the serum albumin value of a patient. These findings are of interest for all drugs with a high albumin binding such as immunosuppressants, antibiotics, or anticancer drugs.



## 1. INTRODUCTION

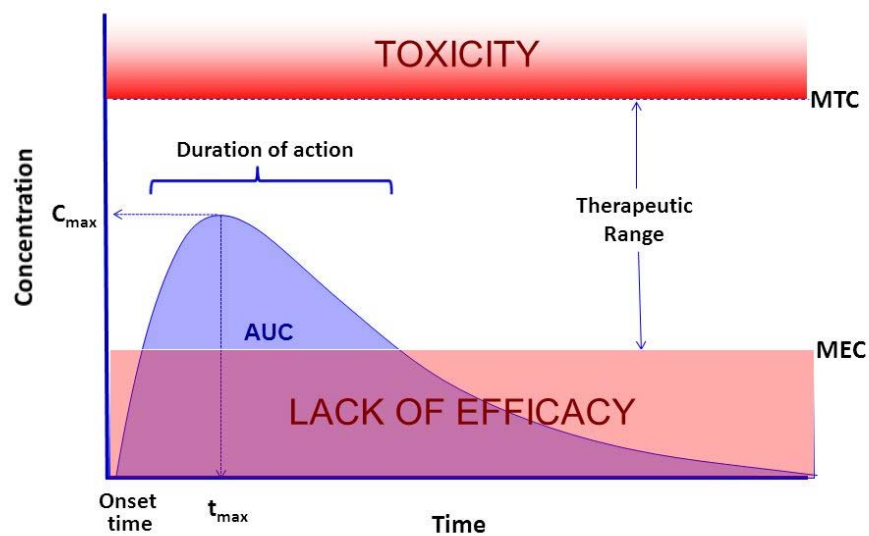
### 1.1 Therapeutic Drug Monitoring

#### 1.1.1 *Factors that Indicate a Therapeutic Drug Monitoring*

Therapeutic drug monitoring (TDM), as a multi-disciplinary clinical approach, has gained wide popularity among clinicians and is aimed at improving patient care by monitoring drug concentrations. There are certain characteristics and criteria which make a range of drugs suitable for or even strongly require a TDM. The monitoring of drug concentrations is, for example, indicated if the pharmacological effect of the drug is clinically not well determinable, if the drug has a narrow therapeutic range, or in the case of insufficient success of a therapy despite therapeutic dosage. For a rational TDM, the drug needs to have a clear relationship between its concentration in different body compartments and its clinical effect or toxicity [1]. Drugs with these characteristics and criteria include, for example, antiepileptics, antibiotics, antimycotics, aminoglycosides, antidepressants, or immunosuppressants. For these drugs, an appropriate TDM becomes highly relevant for a safe and efficient drug treatment. As an example, TDM can make a significant contribution in the context of antiepileptic drug treatments (e.g., phenytoin). A TDM of antiepileptics is of great benefit for the treatment of uncontrollable seizures and in cases of clinical toxicity and assists in the individualization of the therapy and in adjusting for variable or nonlinear pharmacokinetics [2]. An individualized drug therapy (precision medicine) is even more indicated for patients with varying pharmacokinetics, for example, critically ill patients [3], patients with traumatic brain injury, organ failure, very young or elderly patients [4].

Therapeutic Drug Monitoring (TDM) aims to guarantee an optimally individualized drug therapy by maintaining drug concentrations in the specific body compartment

(e.g., plasma) within a targeted therapeutic range. Figure 1 illustrates the relationship between the pharmacokinetics (concentration levels and distribution of drug) and the pharmacodynamics (therapeutic and adverse effects) resulting in the therapeutic range which lies between the maximal therapeutic concentration and the minimal effective concentration.



**Figure 1: Relationship between the Pharmacokinetics and Pharmacodynamics of a Single Dose Administration [5]**

MTC = Maximal therapeutic concentration, MEC = Minimal Effective Concentration,  
AUC = Area Under the Curve

### 1.1.2 Determination of Drug Concentrations for TDM

When monitoring an individualized drug therapy in patients or investigating drug concentrations in forensic toxicology sensitive and specific quantification methods are of critical importance [6]. Critical dose drugs but also newly developed and designed complex drugs require new analytical methods to check for effective drug delivery to target tissues and to minimize toxicity in sensitive organs or cells.

In a hospital setting, an adequate analysis time is crucial for an appropriate TDM and an individualized drug dosing. Bedside analysis is therefore the most appropriate way to measure individual drug concentrations and to swiftly adapt the dose regimen or treatment. For bedside analysis usually homogenous enzyme immune tests (EMIT) are used, which are often sufficient for the determination of drug plasma concentrations and dosage adjustments. For low concentrations in other body compartments, however, these EMITs are not suited.

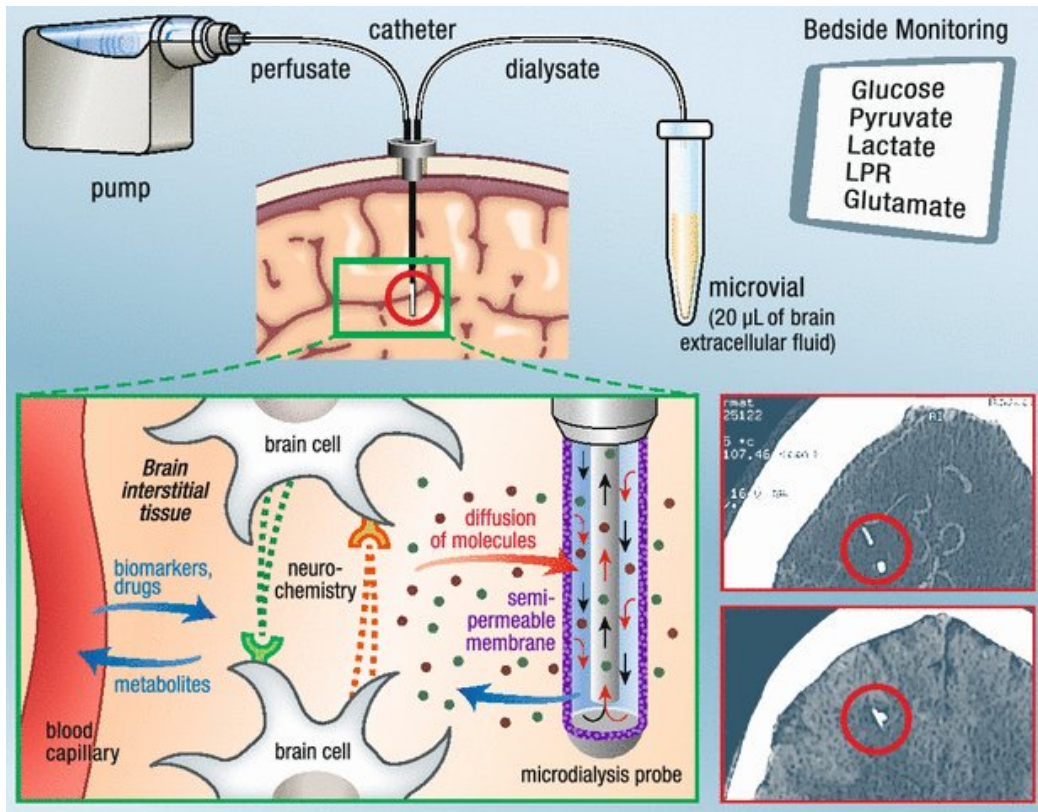
A more precise and sensitive method to measure drug concentrations in different body compartments and already at very low concentrations is the liquid chromatography combined with mass spectrometry (LC-MS or LC-MS/MS). The recent growth in the availability of LC-MS instruments in many hospitals enables to measure drug concentrations on site and allows to react quickly for an adjusted and efficacious drug therapy. The sample volume needed for the analysis with LC-MS method is very small (5-20 $\mu$ l). Therefore, it is possible to measure drug concentrations even in less accessible body compartments as liquor or brain micro dialysates or in small (pre-term) infants.

### *1.1.3 Microdialysis as a Sampling Method in Brain Tissue*

Of the existing drugs in Switzerland in 2017, medications to treat diseases of the central nervous system such as painkillers and drugs to treat Parkinson's disease, mental illness, and epilepsy had the highest market share (16.3%) [7]. The use of antiepileptic drugs has increased during the last decades. In Switzerland, two third of the 70-80'000 affected patients are seizure free due to appropriate antiepileptic medication [8] (see also chapter 1.2.1 of this dissertation).

For antiepileptic drugs such as Phenytoin (PHT), the brain is the site of action and therefore the compartment of interest for an accurate determination of the drug concentration. Brain microdialysis has been established – especially in neurosurgical patients to monitor the status and the metabolism with selected biomarkers or substrates – for sampling and collecting small-molecular-weight substances from the interstitial tissue such as glucose, pyruvate, lactate, LPR, or glutamate. Microdialysis (MD) therefore also enables to collect drugs passing the blood-brain barrier and acting in the brain [9]. MD is a minimally invasive neuro-monitoring technique which uses the principle of dialysis. In Figure 2 the principles of MD are depicted. The microdialysis catheter consists of two concentric tubes. The perfusate flows through the external tube down to the tip (red circle), where the final 10 mm of the catheter consists of a semipermeable dialysis membrane. The substances present in higher concentrations in the brain interstitial tissue diffuse into the dialysis perfusion fluid (red arrow). This perfusate now called MD can then be collected in microvials for analyses. The direction of movement can be changed by adding substances to the perfusion fluid (blue arrow) [10, 11].

An important advantage of the MD method is that it does not affect the compartment volume and therefore the drug concentrations in this compartment do not vary. Furthermore, MD allows a continuous sampling and a nearly unlimited number of samples could be collected. The range of the flow rate for the perfusion fluid can be set from 0.1 - 3  $\mu\text{l}/\text{min}$ . Usually, the perfusion fluid rate is set to 2  $\mu\text{l}/\text{min}$  resulting in collection times between 10 and 12.5 min for 20 - 25  $\mu\text{l}$  sample volume. Therefore, the sampling is rather fast and makes the MD also suitable for bedside drug analysis for TDM and timely dose adjustment.



**Figure 2: Bedside Cerebral Microdialysis Monitoring [10]**

## 1.2 Therapeutic Drug Monitoring of Critical Dose Drugs: The Example of Phenytoin

### 1.2.1 Phenytoin and its Use in Medicine

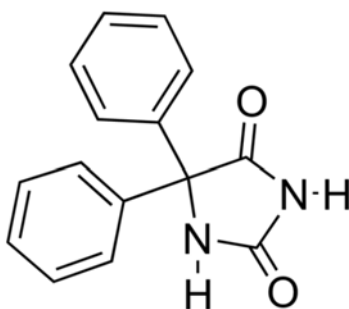
Epilepsy is one of the most important neurological disorders. About 50 million people (1% of the world population) worldwide are affected [12,13]. The annual incidence ranges from 20 to 70 cases per 100'000 and the prevalence is 0.4 to 0.8% [10]. Generalized epilepsies occur in approximately one-third of patients [12]. The treatment of epilepsy is based on stopping seizures or minimizing their frequency as well as mitigating undesirable side-effects [13].

Phenytoin (PHT) is a well-established and one of the most widely prescribed anticonvulsants for the treatment and the prevention of seizures and status

epilepticus [14]. The use of PHT in adults with severe traumatic brain injuries before and after neurosurgical intervention has been shown to be effective as prophylaxis. The risk for an early posttraumatic seizure after acute, traumatic brain injuries can be diminished significantly [15,16,17,18]. The use of antiepileptic drugs to treat patients who have developed post-traumatic epilepsy is common practice [19]. PHT is the only antiepileptic drug for which an optimal therapeutic range (serum concentrations) is clearly defined and which is effective in preventing early seizures after acute brain injury [15, 20].

### 1.2.2 Chemical and Physical Characteristics of Phenytoin

PHT is a hydantoin derivative (5,5-Diphenylhydantoin) with the structure formula  $C_{15}H_{12}N_2O_2$  and the molecular weight of 252.3 g/mol (see Figure 3). PHT is nearly insoluble in water and ethanol 96%, but is soluble in alkaline solutions because of the weak acidic character ( $pK_a = 8.33$ ) [21, 22]. In contrast, PHT-sodium salt (4-oxo-5,5-Diphenyl-4,5-dihydro-1H-imidazol-2-olat)  $C_{15}H_{11}N_2NaO_2$  has a molecular weight of 274.2 g/mol and is soluble in water as well as in ethanol 96% [23]. PHT is a white, crystalline, odorless, and flavorless powder. The sodium salt is also white, crystalline, and lightly hygroscopic [24].



**Figure 3: Chemical Structure of PHT [25,26]**

### *1.2.3 Pharmacokinetics and Pharmacodynamics of Phenytoin*

The therapeutic range of PHT has been defined between 40-80  $\mu\text{mol/L}$  (10-20  $\mu\text{g/mL}$ ). As most drugs, PHT is highly bound to serum proteins. More specifically, PHT has a protein binding (especially to albumin) of 85-95%. Thus, in the case of PHT, only 5-15% unbound or free drug is pharmacologically active [27]. The absolute oral bioavailability of PHT is 85% [28] and the volume of distribution in the body is 0.8 L/kg [29]. PHT has a nonlinear, saturable Michaelis-Menten kinetic; that means the half-life increases with larger but still therapeutic doses. PHT shows a dose dependent half-life of more than 30 hours [30] for therapeutic doses and a clearance ranging from 0.016 to 0.042 L/kg/h [24]. The antiepileptic effect of PHT results from the voltage-dependent blockade of the sodium channels in the neuronal cell-membrane. Thus, rapid potentials along the axons can be interrupted and repeated unloading can be suppressed [24, 31]. Characteristically for the effect of PHT is the dependence of the action from the opening-probability of a channel (so called use-dependence) [32].

### *1.2.4 Metabolism of Phenytoin*

PHT is metabolised nearly completely in the liver. Only about 5% of a dosage is excreted unchanged and the metabolites have no important anticonvulsive effect [33, 34]. PHT is a substrate of the cytochrome P450 (CYP) superfamily, especially of the CYP 2C19 and CYP 2C9 subfamilies, and is therefore subject to various genetic polymorphisms. In the world's population exist more than 10 different mutations of CYP 2C19, resulting in poor metabolisers and extensive metabolisers [35]. There can be up to a twofold difference in the hepatic elimination rate, which leads to different PHT concentrations. Furthermore, the frequencies of different mutations vary among

ethnic populations, so that experiences in the dosage of PHT cannot directly be transferred from one population to another [36].

#### *1.2.5 Suitability of Phenytoin as a Reference Substance for TDM*

The inter-individual genetic differences in the metabolism of PHT combined with the narrow therapeutic range of PHT, non-linear, saturable Michaelis-Menten pharmacokinetics, and long-term therapy as well as relevant interactions with other drugs require a therapeutic drug monitoring (TDM) for adequate PHT dosing. PHT shows a high correlation between drug plasma level and drug efficacy or toxicity [37] and hence fulfills the key prerequisite for a useful TDM.

To maintain an efficacious and safe longtime therapy with PHT, the control of the PHT serum levels in treated patients and the resulting dose adjustments are nowadays standard. An effective TDM depends on rapid, sensitive, specific, and reliable analytical techniques. 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 [27]. 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 [27, 38, 39]. In some cases, however, an estimation of the unbound, free fraction of PHT is mandatory (e.g., in unstable ICU patients) for an appropriate dosing decision (see also chapter 3.3 of this dissertation).



To summarize, the high level of evidence for a TDM of PHT and its complex characteristics make this antiepileptic drug a highly suitable model substance and a reliable standard and comparator for the development and application of TDM for other medications with less evidence.

## 2. AIMS OF THE DISSERTATION

An accurate TDM of critical dose drugs is essential in order to achieve maximum efficacy of the drug as well as to avoid drug toxicity. Critical dose drugs are often used to treat critically ill patients in unstable conditions with disturbed physiological functions and tissue barriers (e.g., neurosurgery patients). For these patients a TDM and the resulting individualized drug therapy is highly beneficial, which has also been demonstrated in precision medicine that tries to optimally use the therapeutic window of a medication [1].

As mentioned above, a minimal invasive sample collecting technique and sensitive, specific, and reliable analytical methods are crucial when studying the pharmacokinetics and pharmacodynamics in humans. Thus, the overall aim of this dissertation is to develop and validate such analytical methods to determine drug concentrations in different body compartments. Thereby, the development, validation, and comparison of different analytical methods is performed by analyzing PHT, which serves as a highly suitable model substance for understanding and improving TDM. PHT is an important substance to prevent complications (i.e., seizures) in neurosurgery patients. Its plasma levels vary to a great extent because of highly interindividual, partly genetically determined differences in metabolism (see also 1.2.4). As mentioned, PHT shows a narrow therapeutic range and a high correlation between the concentration in different body compartments and its clinical effect or toxicity. Therefore, PHT serves as a suitable model substance, where correlating plasma levels with tissue levels and comparing concentrations with easy accessible probes such as saliva samples is possible. Based on the example of PHT, the findings

may serve as a foundation for the TDM of other substances and the methods may be extrapolated across different drugs.

The common method to measure total PHT in plasma concentrations is a homogenous enzyme immune test (e.g., Syva corporation; Siemens Medical Devices) [40]. Homogenous enzyme immune tests (EMIT) have been shown to be sufficient for most simple dosage adjustments (based on a classical TDM using plasma samples), as PHT shows a high correlation between total drug plasma level and drug efficacy or toxicity [37]. These commonly used methods, however, do not meet the requirements for more sophisticated pharmacological investigations such as pharmacokinetic studies in different body compartments (e.g., brain microdialysates), correlation studies between the PHT concentration in blood plasma and the site of action, or the quantification of the free PHT fraction – mostly due to insufficient sensitivity and missing validation in other sample matrices [41].

Researchers and practitioners are interested in specific, sensitive, robust, and cost-effective methods to identify PHT concentrations. Thus, a first objective of the present dissertation was to establish a highly selective and sensitive GC-MS method which allows the determination of PHT in different human biological samples, especially in brain microdialysates. The first publication deals with this research question [42].

Recently, also LC-MS/MS methods have become commonly available in routine medical labs for measuring drug concentrations, as they offer some advantages over other sensitive methods such as GC-MS [43]. Therefore, a second objective of the dissertation was to develop and validate a LC-MS/MS method for the measurement of PHT concentrations in different body compartments such as blood and saliva as

well as samples from brain tissue microdialysis often clinically used in neurology and neurosurgery, and to compare its performance with established, validated methods (e.g., GC-MS [42]). These further investigations are documented in the second publication [44].

As there are not always the required resources in hospitals for the necessary sample processing and for specific, demanding lab testing instruments such as GC-MS or LC-MS/MS units to measure the free unbound fraction of a drug, a third objective was to calculate the free fraction of PHT from conventional total drug TDM with EMIT using the Sheiner-Tozer equation and individual albumine levels. Thereby, the estimated values have to be compared with measured concentrations in samples from patients of different ages and with different diseases – factors which are known to influence the free drug fraction [27]. In such cases, the knowledge of the free PHT fraction would be useful in TDM and lead to improved dosing decisions. This objective is addressed in the third publication [45].

### 3. METHODS AND RESULTS

According to the outlined objectives, the dissertation is based on three PubMed cited peer-reviewed publications, which are included in their specific journal layout in chapters 3.1 – 3.3 of this dissertation.

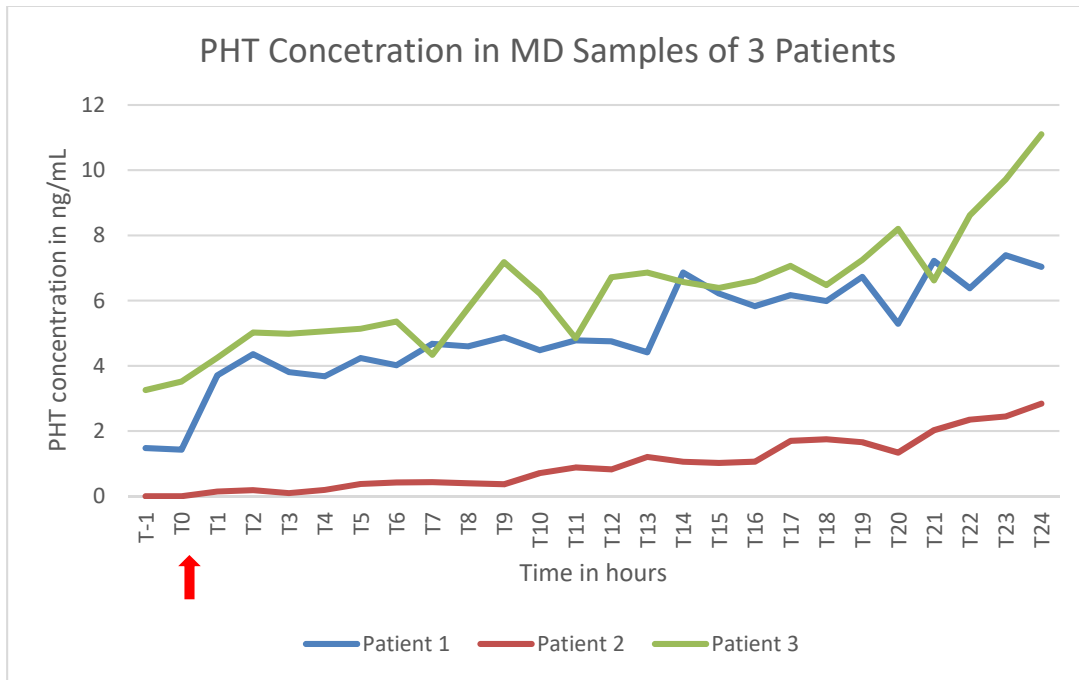
The first article describes the development and validation of a gas chromatography–mass spectrometry (GC–MS) method to identify and quantitate PHT in brain microdialysate, saliva, and blood from human samples. A solid-phase extraction (SPE) with a nonpolar C8-SCX column was used for sample clean-up and PHT extraction. The eluate was evaporated with nitrogen (50°C) and derivatized with trimethylsulfonium hydroxide before GC–MS analysis. The internal standard was 5-(p-methylphenyl)-5-phenylhydantoin. 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  $\geq 94\%$ . 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 (LOD) was 15 ng/mL and the limit of quantification (LOQ) was 50 ng/mL. Dried extracted samples were stable within a 15% deviation range for  $\geq 4$  weeks at room temperature. The method met ISO 17025 standards and was able to detect and quantify PHT in different biological matrices and patient samples.

The second article describes a newly developed LC-MS/MS method for the measurement of PHT concentrations in different body tissues (i.e., human brain dialysate, blood, and saliva) and compares it with the GC-MS method [42]. The two methods are evaluated and compared based on their analytical performance,

appropriateness to analyze human biological samples, including corresponding extraction and cleanup procedures, and their validation according to ISO 17025 and FDA Guidance for Industry.

The LC-MS/MS was more sensitive, needed a smaller sample volume (25  $\mu$ L) and less chemicals, was less time consuming (cleaning up, sample preparation, and analysis), and resulted in a 15 times better LOD (<1 ng/mL) and 5 times better LOQ (10 ng/mL). The calibration curve of the LC-MS/MS method showed linearity over a larger range (i.e., 10–2000 ng/mL compared to 50–1200 ng/mL for the GC-MS) with correlation coefficients  $r^2 > 0.995$  for all tested matrices (blood, saliva, and dialysate). The investigation indicates that for larger sample numbers as in clinical or non-clinical pharmacokinetic / pharmacodynamics studies and for bedside as well as routine analyses, the LC-MS/MS method offers significant advantages over the GC-MS method.

In addition to the published results, the LC-MS/MS method was further used for analyzing blinded MD samples from three patients with a cranial trauma. Each patient received 125 mg PHT as an intravenous injection at time  $t_0$  (red arrow). Samples were then collected and analyzed hourly (for 24h) (see Figure 4). As depicted in the figure, the PHT concentrations in the MD of the patients varied between 0 and 12 ng/mL and increased differently (with a 24hrs peak value between 3 and 11 ng/mL). This illustrates that this LC-MS/MS method is able to determine very low PHT concentration levels. The limit of detection of the GC-MS method was 15 ng/mL and therefore could not be used for these patient samples.



**Figure 4: Measured PHT Concentrations in MD Samples from three Patients with Cranial Trauma**

To further illustrate the suitability of the LC-MS/MS method for measuring drug concentrations in MDs we have also analyzed another antiepileptic drug (i.e., levetiracetam). In a first test, we were able to accurately measure levetiracetam concentrations in blinded MD samples of 4 patients with cranial trauma using an existing LC-MS/MS method at the Institute of Forensic Toxicology and Chemistry, University of Bern (see Appendix A). The application of levetiracetam in these patients was off label since levetiracetam was given to prevent seizures after neurosurgical interventions. Thus, these measurements could be of use to understand the pharmacokinetics / pharmacodynamics of this drug in this specific patient population and could help physicians to optimize the therapy.

Finally, the third article investigated the suitability of the Sheiner-Tozer algorithm to estimate the free fraction of PHT (> 90% albumin binding) in hypoalbuminemic patients. Free PHT plasma concentration was calculated from total PHT concentration and compared with the measured free PHT of 23 patients. The patients were separated into a low ( $35 \leq \text{albumin} \leq 25 \text{ g/L}$ ) and a very low group ( $\text{albumin} < 25 \text{ g/L}$ ) for comparing and statistically analyzing the calculated and the measured free PHT concentration.

The calculated (1.2 mg/L; SD = 0.7) and the measured (1.1 mg/L; SD = 0.5) free PHT concentration correlated. The mean differences in the low and the very low albumin group were: 0.10 mg/L (SD = 1.4) (n = 11) and 0.13 mg/L (SD = 0.24) (n = 12), respectively. No statistically significant differences between the groups were found. 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)].



## ARTICLE 1

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

Raphael Hösli, Andrea Tobler, Stefan König, and 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  $\geq 94\%$ . 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  $\geq 4$  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) [C<sub>15</sub>H<sub>12</sub>N<sub>2</sub>O<sub>2</sub> (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  $\mu$ mol/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 quantitate 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  $\mu$ L used in a recently published study is far too large for the usual microdialysis sampling in patients, with a 2  $\mu$ L/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 (C<sub>16</sub>H<sub>14</sub>N<sub>2</sub>O<sub>2</sub>; 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<sub>16</sub>H<sub>14</sub>N<sub>2</sub>O<sub>2</sub>; 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<sub>2</sub> and 0.1758 g MgCl<sub>2</sub> × 6(H<sub>2</sub>O), 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 µL min, yielding a sample of approximately 60 µL. 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°C. For transportation, a refrigeration chain (–20 ± 2°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°C. This stock solution was diluted with MeOH to 100 ng/µL for addition to solutions [internal standard solution (ISS), 100 ng/µL]. The PHT reference stock solution containing 1 mg PHT/mL MeOH was stored at 2–8°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 µL 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 µL 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 µL ISS).

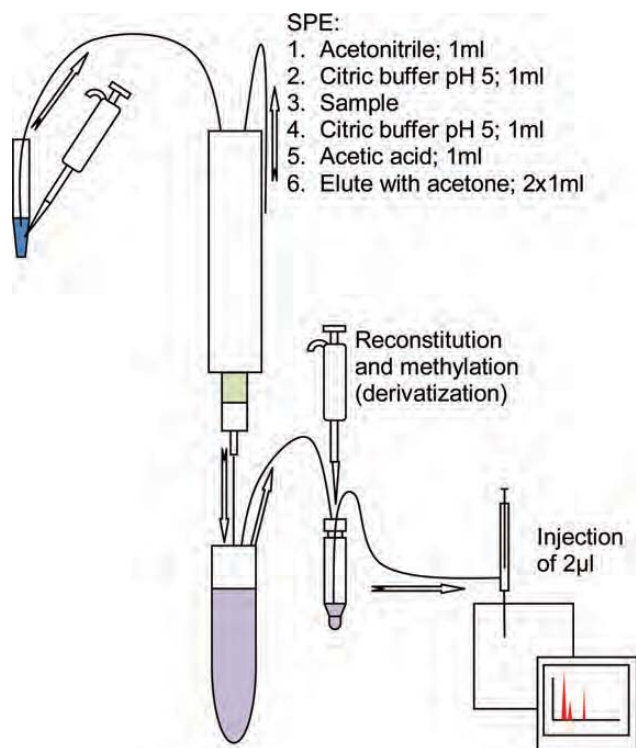
Samples from patients were included to test the method in real biological probes. PHT-containing microdialysis samples (volume of 50 µL) from six patients (requiring approximately 25 min of collection time) were added to 450 µL aCSF and 1,200 ng MPPH (12 µL ISS) as IS prior to extraction. Blood plasma samples (volume of 500 µL) from PHT-treated patients were spiked with 1,200 ng MPPH (12 µL 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 ~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°C. The reconstitution and derivatization were performed with 50 µL 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 µL 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 µm; for temperatures from –60 to 350°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.

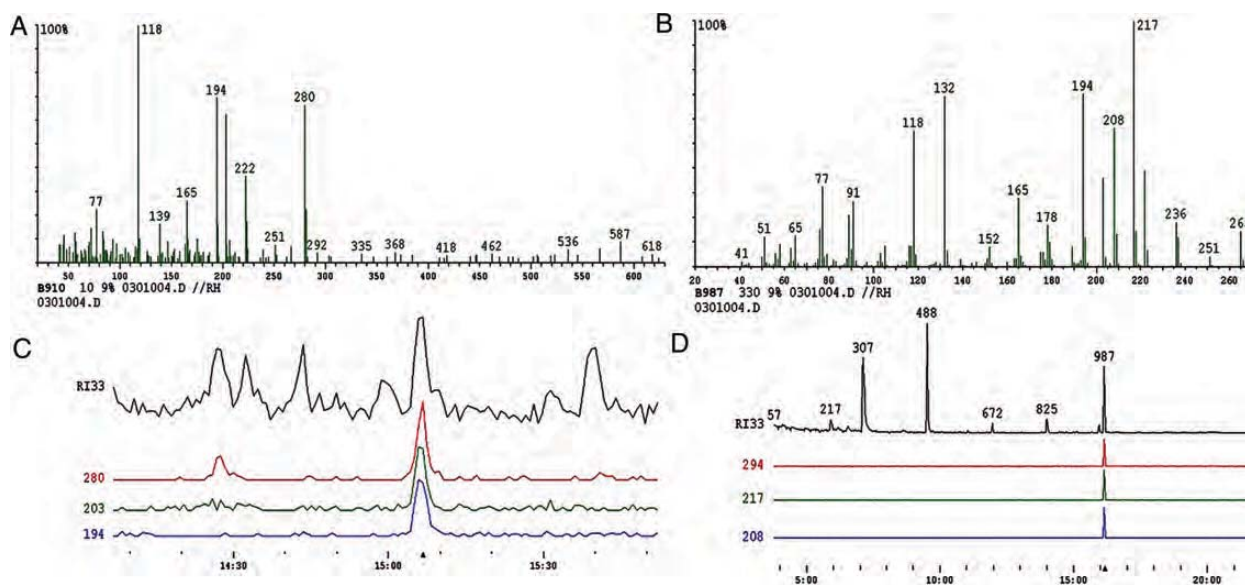
#### Results and Discussion

##### 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 D). 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 D). 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 D).

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\*

Sample name	Overall mean	SD	Mean deviation (%)	Inter-assay CV (%)	Accuracy (%)
Calculated data from the measurements with aCSF as matrix					
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	Saliva: mean/ deviation from target concentration	Blood plasma: mean deviation from target concentration		
	(n = 6)	(n = 3)	(n = 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 ( $r^2$ )	0.999	0.999	0.998		
y-intercept	0.288	3.818	5.542		
Slope (x) 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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## **ARTICLE 2**

### **Development and Validation of an LC-MS/MS Method and Comparison with a GC-MS Method to Measure Phenytoin in Human Brain Dialysate, Blood, and Saliva**

Raphael Hösli, Stefan König, and Stefan F. Mühlebach

## Research Article

# Development and Validation of an LC-MS/MS Method and Comparison with a GC-MS Method to Measure Phenytoin in Human Brain Dialysate, Blood, and Saliva

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Phenytoin (PHT) is one of the most often used critical dose drugs, where insufficient or excessive dosing can have severe consequences such as seizures or toxicity. Thus, the monitoring and precise measuring of PHT concentrations in patients is crucial. This study develops and validates an LC-MS/MS method for the measurement of phenytoin concentrations in different body compartments (i.e., human brain dialysate, blood, and saliva) and compares it with a formerly developed GC-MS method that measures PHT in the same biological matrices. The two methods are evaluated and compared based on their analytical performance, appropriateness to analyze human biological samples, including corresponding extraction and cleanup procedures, and their validation according to ISO 17025/FDA Guidance for Industry. The LC-MS/MS method showed a higher performance compared with the GC-MS method. The LC-MS/MS was more sensitive, needed a smaller sample volume (25  $\mu$ L) and less chemicals, was less time consuming (cleaning up, sample preparation, and analysis), and resulted in a better LOD (<1 ng/mL)/LOQ (10 ng/mL). The calibration curve of the LC-MS/MS method (10–2000 ng/mL) showed linearity over a larger range with correlation coefficients  $r^2 > 0.995$  for all tested matrices (blood, saliva, and dialysate). For larger sample numbers as in pharmacokinetic/pharmacodynamic studies and for bedside as well as routine analyses, the LC-MS/MS method offers significant advantages over the GC-MS method.

## 1. Introduction

Sensitive and specific quantification methods are of critical importance when monitoring individualized drug therapy in patients or investigating drug concentration in forensic toxicology [1]. Critical dose drugs but also newly developed and designed complex drugs require analytical methods to check for effective drug delivery to target tissues and to minimize toxicity in sensitive organs or cells. When such drugs have to be used in patients with varying pharmacokinetics (PK) (e.g., ICU patients), an appropriate therapeutic drug monitoring (TDM), which allows, for example, to correlate the drug concentration in easy accessible plasma samples with those in the tissue of action, becomes even more relevant for a safe and efficient drug treatment [2].

Phenytoin (PHT) belongs to the most widely prescribed drugs to prevent and control most types of seizure disorders and to treat epilepsy [3]. It is one of the most often used critical dose drugs where insufficient or excessive dosing can have severe consequences such as seizures or toxicity. Thus, the monitoring and precise measuring of PHT concentrations in patients is crucial [4, 5]. As an example, in forensic toxicology, epilepsy patients under PHT treatment who have been involved in an accident have to be analyzed in order to verify whether the PHT concentration was adequate or possibly the reason for the accident [6]. However, there are several characteristics of PHT including a relatively low therapeutic index, difficult pharmacokinetics (PK) and pharmacodynamics (PD), saturable oxidative biotransformation, and the nonlinear clearance, which complicate a therapeutic

drug monitoring (TDM) aimed at preventing intoxication of patients or treatment failures [7].

Thus, researchers and practitioners are interested in specific, sensitive, robust, and cost-effective methods to identify PHT concentrations in patients. Thereby, several compartments to measure the PHT concentration could be addressed such as blood, saliva, and CNS fluid (microdialysate). The correlation of PHT in different body compartments is not yet completely understood and has only recently been addressed by researchers who have compared the measurement of PHT in these different compartments with a GC-MS method [8]. While the GC-MS has long been the standard method in forensic testing, LC-MS/MS methods have become more common, as they generally offer some advantages over GC-MS [9]. Recently, researchers have developed an LC-MS/MS method to measure PHT in one specific body compartment (i.e., blood plasma or serum) [10]. Missing, however, is a thorough comparison of the performance of these two analytical methods in the detection and analysis of PHT in different body compartments (i.e., blood, saliva, and samples from brain tissue microdialysis).

The aim of the present study was to develop and validate an LC-MS/MS method for the measurement of PHT concentrations in different body compartments such as blood and saliva, as well as samples from brain tissue microdialysis often used in neurology and neurosurgery, where antiepileptic therapy is often mandatory [11, 12], and to compare its efficiency with a formerly developed GC-MS method [8]. The fact that this established GC-MS method measured PHT in the same biological matrices (i.e., blood, saliva, and human brain dialysate) enables a reliable comparison with regard to the performance of GC-MS versus LC-MS/MS in measuring PHT in different body compartments. The two methods are evaluated and compared based on their analytical performance, appropriateness to analyze human biological samples, including corresponding extraction and cleanup procedures, and their validation according to ISO 17025/FDA Guidance for Industry [13]. Finally, the suitability of the two analytical methods for PK/PD studies, bedside measurement, and forensic use is discussed. In addition, the LC-MS/MS method developed in the current study is compared with an established LC-MS/MS method which measured PHT in blood plasma samples [10].

## 2. Materials and Method

**2.1. Chemicals and Samples Used for the Development of the LC-MS/MS Method and Its Validation.** PHT reference substance was purchased from Desitin Pharma GmbH (Liestal, Switzerland) and from the European Pharmacopoeia (PHT Ph. Eur. Standard, EDQM, Strasbourg, France). The IS for LC-MS/MS was PHT-D<sub>10</sub> (PHT D-10, C<sub>15</sub>H<sub>2</sub>D<sub>10</sub>N<sub>2</sub>O<sub>2</sub>, MW = 262.33) in methanol (MeOH) (100 µg/mL) from Cerilliant (Round Rock, TX).

Calcium chloride, perchloric acid, citric acid monohydrate, potassium chloride, magnesium chloride hexahydrate, sodium chloride, sodium hydroxide, and the solvents (methanol, acetic acid 100%, and acetone) were of analytical grade and purchased from Merck (Darmstadt, Germany).

Artificial cerebrospinal fluid (aCSF; dialysate solution) was prepared according to M Dialysis AB (Stockholm, Sweden) [14]. Blood CPDA-1 (anticoagulant citrate phosphate dextrose adenine solution; to simplify only named blood in the following) was obtained from the Blood Donor Center (Bern, Switzerland). Saliva was obtained from one of the investigators. 20–60 µL PHT-containing samples from patients collected from a 2 µL/min flow rate brain microdialysis and 2 mL of CPDA containing PHT patient blood samples were provided by the Department of Neurosurgery (Kantonsspital Aarau AG, Switzerland and Centre Hospitalier Universitaire Vaudois, Switzerland). All biological samples (blood and dialysates) were frozen and stored at –24°C. Before sample analysis, the samples were thawed at room temperature for 30 minutes and homogenized by shaking with a vortex for one minute.

**2.2. Internal Standards, Calibrator Standard System Suitability Testing, and Sample Preparation.** The internal standard (IS) stock solution was prepared by adding 100 µL of the PHT-D<sub>10</sub> (100 µg/mL) to 9900 µL of MeOH. 5 mL of this solution was added to 95 mL of 1 M perchloric acid aqueous solution to get the final concentration of 50.0 ng/mL, which is used as IS working solution. The PHT reference stock solution (1.00 mg/mL) was used to obtain eight calibration (Cal) solutions with concentrations of 2000, 1000, 500, 250, 100, 50, 20, and 10 ng/mL PHT. 20 µL of these Cal solutions were added to 980 µL of the biomatrices to get the Cal working solutions. For quality control (QC), solutions with 1600, 400, 30, and 10 ng/mL PHT were prepared out of PHT reference stock solution (1.00 mg/mL).

The IS working solution of 75 µL was added either to an aliquot of 25 µL Cal working solution, QC solutions, or 25 µL sample from patients containing PHT. The sample preparation for the LC-MS/MS consisted of pipetting 75 µL of IS working solution to 25 µL sample into a deep well plate (0.6 mL, Chemie Brunschwig AG, Basel, Switzerland) covered by a sealing mat (Silicone, Chemie Brunschwig AG, Basel, Switzerland). The well plates were rigorously shaken for 5 minutes and then centrifuged (4.500 U/min; Mikro 22R, Hettich Instruments, Andreas Hettich AG, Bäch, Switzerland) for 30 minutes at about 8°C (Figure 1). The processed samples were ready for the LC-MS/MS analysis.

**2.3. LC-MS/MS Settings.** The prepared samples were placed into the autosampler (Dionex WPS-3000TSL Olten, Switzerland) which was set at 8°C. With a 100 µL syringe from the autosampler, 10 µL of the prepared samples was injected into a 130 µL loop. The solvent rack (Dionex SRD-3600, Olten, Switzerland) carried the mobile phase A (H<sub>2</sub>O + HCOOH (100 + 0.1, v + v)) and phase B (MeCN + HCOOH (100 + 0.1, v + v)). These mobile phases were delivered by three pumps (binary pump 1 (flow 0.350 mL/min) and isocratic pump 2 (flow 0.200 to 1.000 mL/min) (Dionex pump HPG-3200A, Olten, Switzerland), and binary pump 3 (Dionex pump ISO-3100A, Olten, Switzerland)) connected to a triple stage quadrupole mass spectrometer with linear ion-trap capability (3200 QTrap, Analyst Software Version 1.5.1, Applied

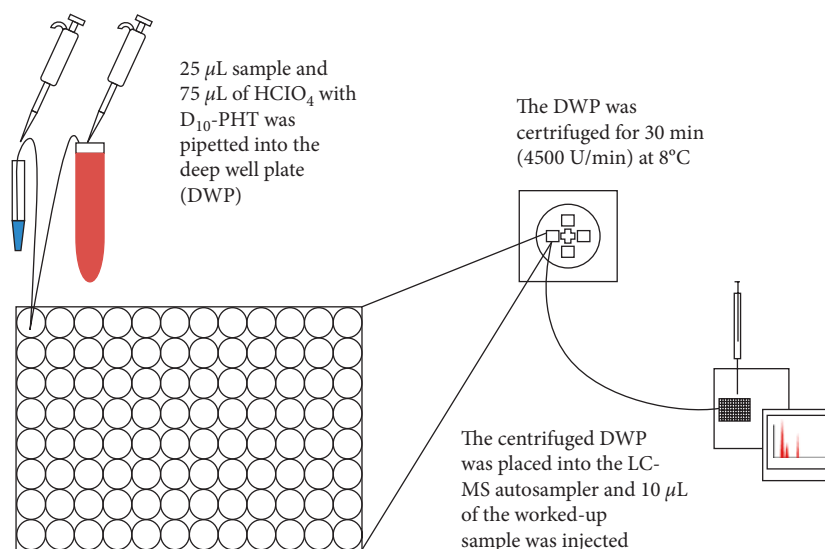


FIGURE 1: Sample preparation for the LC-MS/MS analyses for blood, saliva, and aCSF samples.

TABLE 1: Settings of the HPLC program.

Time (minutes)	Pump 1 (main column (MC))			Pumps 2 and 3 (trapping column (TC))			
	%B	Flow ( $\mu\text{L}/\text{min}$ )	Comments	%B	Flow ( $\mu\text{L}/\text{min}$ )	Flow pump 5 ( $\text{H}_2\text{O} + 0.1\% \text{HCOOH}$ ) ( $\mu\text{L}/\text{min}$ )	Switching valve
0	35		Start MS and pumps	50	300	800	TC $\rightarrow$ waste, MC $\rightarrow$ MS (loading)
0.5	35		Start gradient	50	300	800	TC $\rightarrow$ MC $\rightarrow$ MS (eluting)
0.6	$\downarrow$						
1	97.5	500		50	20	20	
2	97.5						TC $\rightarrow$ waste, MC $\rightarrow$ MS
2.5	35			50	300	800	Reequilibration

Biosystems/MDS Sciex, Toronto, Canada) (Table 1). For the mass spectrometric detection, SRM scan mode (selective reaction monitoring) was used. SRM transitions and mass spectrometric conditions were as follows: transition: 253.1  $\rightarrow$  182.2 (PHT) and 253.1  $\rightarrow$  192.2 (PHT- $\text{D}_{10}$ ); orifice (V): 36; collision energy (eV): 41 (PHT) and 51 (PHT- $\text{D}_{10}$ ); and dwell time (msec): 100. Electrospray ionization was performed in positive ion mode for the analyte and the IS. The following instrument parameters for ionization were used: ion source voltage: 5000 volt, curtain gas: 25, gas 1: 40 and gas 2: 60; and the CAD gas was set to 5 (arbitrary units for the gas settings). As trapping column, a Phenomenex Gemini Polar column (2.0  $\times$  10 mm, 5  $\mu\text{m}$ ; Brechbühler AG, Schlieren, Switzerland) tempered to room temperature was used. The main column Phenomenex Synergy Polar RP column (2.0  $\times$  50 mm; Brechbühler AG, Schlieren, Switzerland) was placed into the column oven (Cluzeau Info Labo CrocoCil) set on 50°C with a column thermostat (Dionex TCC-3100, Olten, Switzerland) including switching valve (Figure 2). This system was operated by Analyst Software (Version 1.5.1, AB Sciex, Toronto, Canada).

2.4. Validation of the LC-MS/MS Method according to ISO 17025/FDA Guidance for Industry. The validation was carried out according to ISO 17025/FDA Guidance for Industry

including selectivity, sensitivity, accuracy, recovery of PHT, reproducibility and suitability of the calibration curves, stability of PHT, and matrix effects. The selectivity and sensitivity (absence of PHT) were verified by analyzing blank samples without PHT (extraction and matrix effects). For the accuracy, QCs and Cal samples were analyzed. The recovery of PHT was analyzed by measuring QCs at different levels. The reproducibility and suitability of the calibration curves was measured by a complete series of Cal 1 to Cal 8 (LC-MS/MS) analyses. The limit of detection (LOD) and the limit of quantification (LOQ) were analyzed using Cal 1 (LC-MS/MS 10 ng/mL PHT). The LOD was checked as a signal-to-noise (S/N) ratio of more than 4:1. The LOQ was considered as 5 times the response to a blank sample. The stability tests consisted of the freeze-thaw stability of PHT, which was determined after 3 freeze-thaw cycles. The short-term stability was analyzed by keeping the samples thawed at ambient temperature for at least 6 hours, frozen for at least 12 hours at  $-25^\circ\text{C} \pm 5^\circ\text{C}$ , and again thawed, worked-up, and analyzed. Postpreparative stability was evaluated to determine whether an analytical run can be reinjected in the case of instrument failure and, furthermore, whether the preparation of a large number of samples could be done at once. Therefore, one of the validation runs was analyzed a second time after 7 days. The described criteria for Cal curves, QC, accuracy, and precision had to be met.

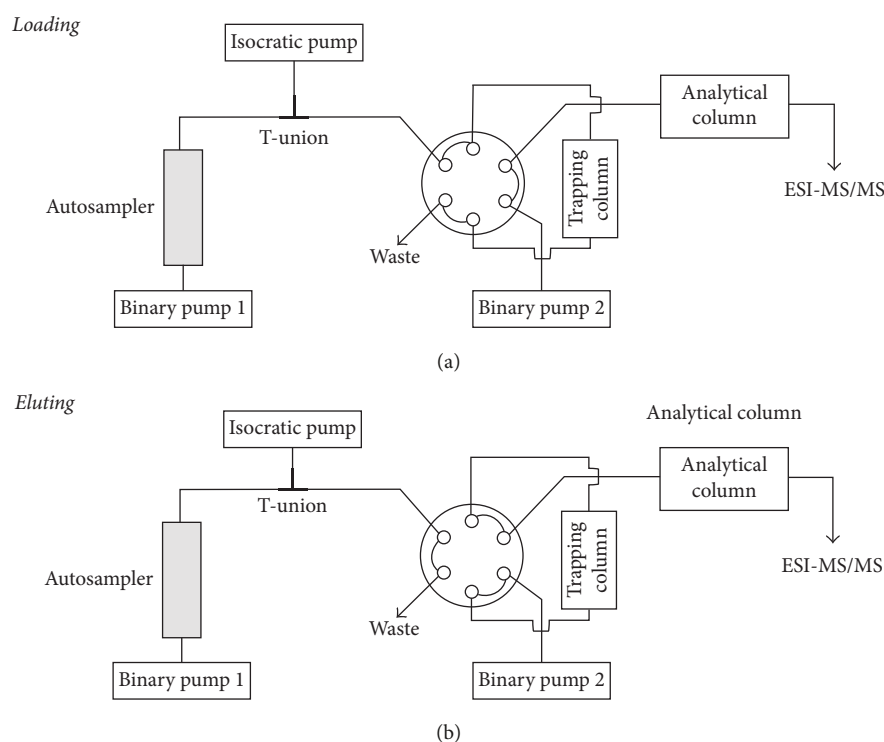


FIGURE 2: LC-MS/MS settings.

Matrix effects were analyzed by comparing the calibration curves generated with the three matrices aCSF, blood, and saliva. PHT microdialysis and blood samples from patients were analyzed to demonstrate the suitability of the method for biological samples from patients.

**2.5. Comparison of the LC-MS/MS and the GC-MS Method.** The LC-MS/MS method was evaluated and compared with the GC-MS method published by Hösli et al. [8] with regard to its analytical performance, appropriateness to analyze human biological samples, including corresponding extraction and cleanup procedures, and its validation according to ISO 17025/FDA Guidance for Industry.

The statistical data were calculated with Microsoft Excel and IBM SPSS Statistics 22. To compare the different matrices, a one-way ANOVA was calculated. The corresponding values were checked for significance by *t*-tests.

### 3. Results

**3.1. Validation of the LC-MS/MS Method.** The retention time (RT) for PHT and for PHT-D<sub>10</sub> (IS) was about 2.8 min (Figure 3). The selectivity and sensitivity were checked; all blank samples were negative. The recovery of PHT after precipitation with HClO<sub>4</sub> was 89.5% for QC1 (10 ng/mL) and 97.1% for QC3 (1600 ng/mL) compared to the amount found in unprepared samples (=100%). The LOD calculated as *S/N* ratio of 4:1 for this method in aCSF, saliva, and blood was set at <1 ng/mL. The LOQ calculated as 5 times the response/blank was 10 ng/mL PHT. For the accuracy, the Cal 1 to Cal 8 were assessed. The calibrator values showed

min-max deviations of 1–8% for Cal 2 (20 ng/mL) to Cal 8 (2000 ng/mL) with 3% for Cal 1 (10 ng/mL). The calibration curves for all three matrices were linear. The regression coefficients ( $r^2$ ) of the three different matrices were  $r^2_{\text{blood}} = 0.996$  ( $n = 3$ ),  $r^2_{\text{dialysate}} = 0.997$  ( $n = 6$ ), and  $r^2_{\text{saliva}} = 0.995$  ( $n = 3$ ). Reinjection after 7 days showed no difference in accuracy. The sample volume needed was 25  $\mu\text{L}$ . The sample preparation time was about 2 min per sample (6 hours for 182 samples). The run time for one LC-MS/MS analyses was 7 min.

**3.2. Comparison of the LC-MS/MS with the GC-MS Method.** After validation of the LC-MS/MS method, it was compared with the referred GC-MS method [8]. Table 2 shows the comparative results of the two methods for their analytical performance, appropriateness to analyze human biological samples, including corresponding extraction and cleanup procedures, and its validation according to ISO 17025/FDA Guidance for Industry (Table 2).

The selectivity and the sensitivity were met by both methods, and the recovery showed no differences (Table 2). But the accuracy differed between the two methods. The GC-MS method showed a higher variation at Cal 1 (20%) than the LC-MS/MS method (Cal 1 = 3%). As expected, the biggest difference in terms of analytical performance between the two methods was observed by the LOQ (GC-MS = 50 ng/mL; LC-MS/MS = 10 ng/mL) and LOD (GC-MS = 15 ng/mL; LC-MS/MS = <1 ng/mL) (Table 2).

Both methods showed linear regression coefficients ( $r^2$ ) higher than 0.995 in all three different matrices for the PHT calibration curve. The calibration range of the LC-MS/MS

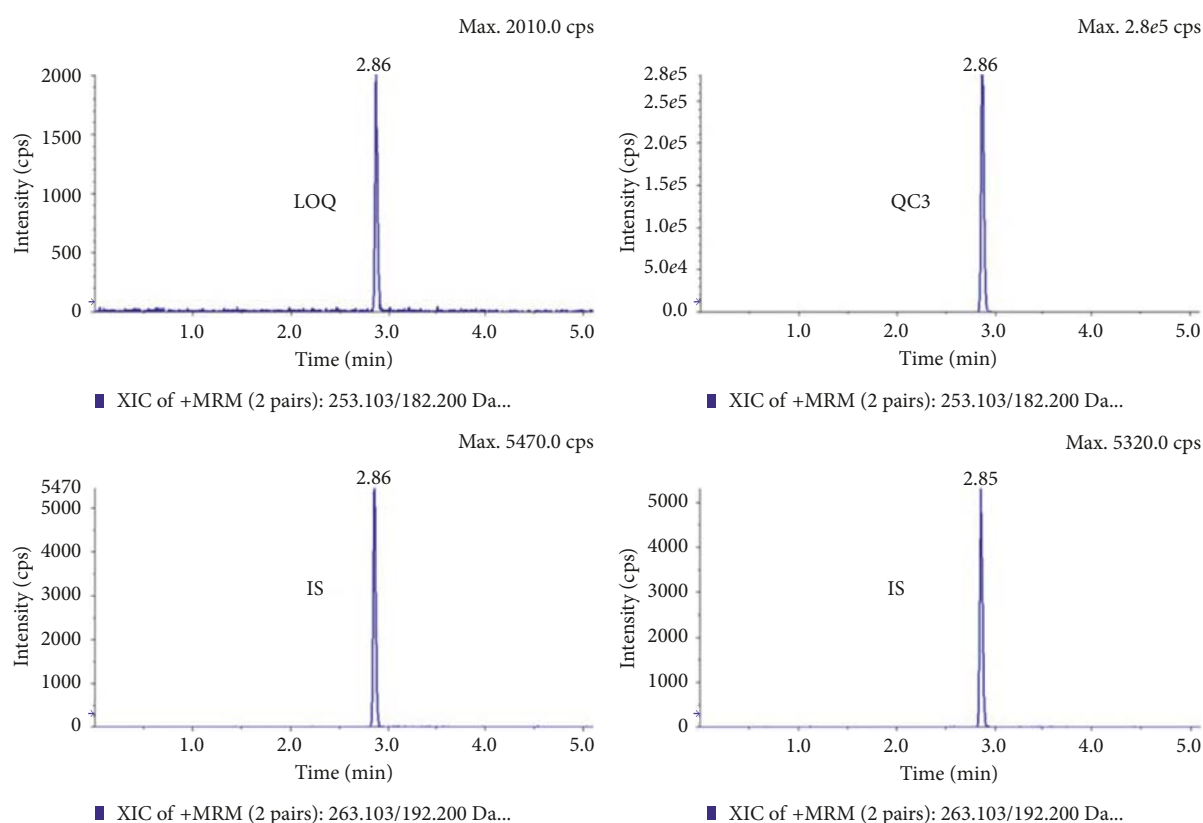


FIGURE 3: Chromatogram of phenytoin (illustrated for LOQ (10 ng/mL) and QC3 (400 ng/mL)) with PHT-D<sub>10</sub> as IS (50 ng/mL).

TABLE 2: Comparison of the GC-MS [8] versus LC-MS/MS method.

Criterion	GC-MS	LC-MS/MS
Retention time	PHT 15.12 min, IS MPPH 16.15 min	PHT and PHT-D <sub>10</sub> 2.8 min
Selectivity/sensitivity (absence of PHT)	Good peak differentiation and quantification of PHT. All blank samples were negative (no presence of PHT)	All blank samples were negative (no presence of PHT)
Recovery	94.1% for QC2 (100 ng/mL) 94.3% for QC5 (1000 ng/mL)	89.5% for QC1 (10 ng/mL) 97.1% for QC3 (1600 ng/mL)
LOD (calculated as S/N ratio of 4:1)	15 ng/mL	<1 ng/mL
LOQ (calculated as 5 times the response/blank)	50 ng/mL	10 ng/mL
Accuracy	The calibrator values showed min–max percent deviations of 1–20% for Cal 1 (50 ng/mL) to Cal 6 (1200 ng/mL)	The calibrator values showed min–max percent deviations of 1–8% for Cal 1 (10 ng/mL) to Cal 8 (2000 ng/mL)
Regression coefficient, $r^2$	$r^2_{\text{blood}} = 0.998$ ( $n = 2$ ) $r^2_{\text{dialysate}} = 0.999$ ( $n = 8$ ) $r^2_{\text{saliva}} = 0.999$ ( $n = 2$ )	$r^2_{\text{blood}} = 0.996$ ( $n = 3$ ) $r^2_{\text{dialysate}} = 0.997$ ( $n = 6$ ) $r^2_{\text{saliva}} = 0.995$ ( $n = 3$ )
Calibration range	50–1200 ng/mL	10–2000 ng/mL
Run time per analysis	30 min	7 min
Injection volume of the sample	2.0 $\mu\text{L}$	10 $\mu\text{L}$
Sample preparation time	5 h for 25 samples	6 h for 182 samples
Stability of the processed samples	Dried extracts were stable for $\geq 4$ weeks (min/max deviation 4%). No effect by reinjection and storage (33 h) on the autosampler	Reinjection after 7 days showed no difference in accuracy
Sample volume needed	50 $\mu\text{L}$	25 $\mu\text{L}$

(from 10 ng/mL to 2000 ng/mL) is twice as large as of the GC-MS (50 ng/mL to 1200 ng/mL). The stability of the samples after extraction and cleaning up was demonstrated

for both methods. The sample preparation procedure is demonstrated in Figure 1 for LC-MS/MS and Figure 4 for GC-MS.

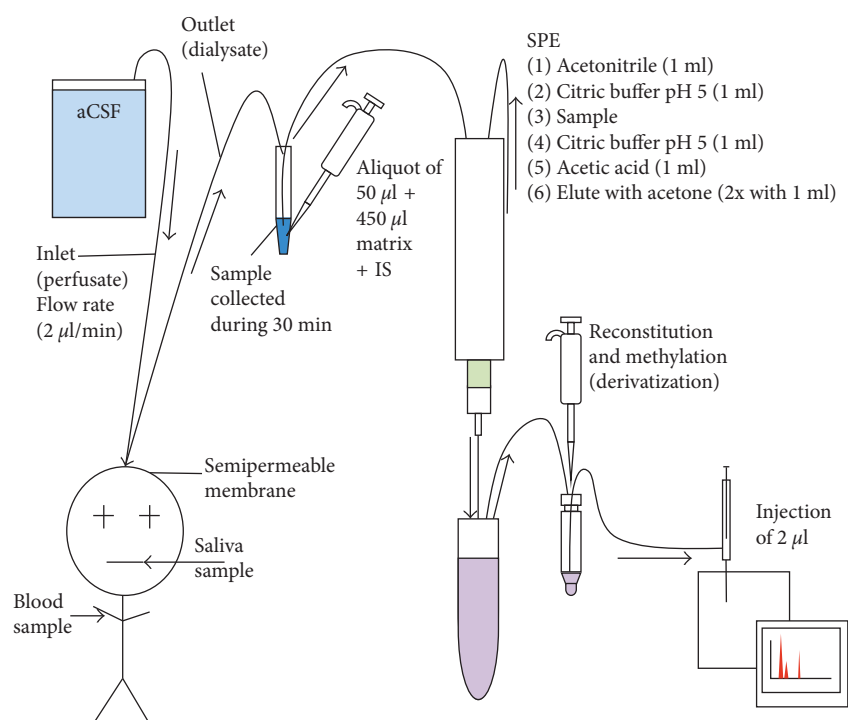


FIGURE 4: Sample preparation for the GC-MS analyses [8].

**3.3. Comparison of the LC-MS/MS Method with a Formerly Established LC-MS/MS Method.** Recently, a LC-MS/MS method has been developed which measures PHT in blood plasma or serum [10]. For the measurement of PHT in blood, the newly validated LC-MS/MS method can hence also be compared with this recently published study. The two methods show some similarities such as an identical IS (100 µg/mL PHT-d<sub>10</sub>), similar sample volumes needed (25 µL versus 20 µL [10]), and a comparable retention time (2.8 min versus approximately 2.1 min [10]). Both methods showed linear regression coefficients ( $r^2$ ) higher than 0.99 in the blood matrix. The accuracy was similar as both studies showed deviations of <10%. With regard to the calibration range and the calibration solution, the two LC-MS/MS methods differ. While the LC-MS/MS method developed in this study showed a calibration range from 10 ng/mL to 2000 ng/mL, the calibration curve of the published LC-MS/MS method [10] ranged from 100 ng/mL to 4000 ng/mL. The calibration solution in the current study was the respective biological matrix (e.g., blood). In the published study [10], phosphate-buffered saline was used as the calibration solution.

#### 4. Discussion

In this study, a LC-MS/MS method to measure PHT in different biological samples was successfully validated and compared with a similarly validated GC-MS method [8]. Overall, the LC-MS/MS method showed to be a more specific analytical method with a higher general performance (Table 2). The LC-MS/MS method needed less sample volume, less chemicals, and less analytical time and therefore resulted in less costs for the sample preparation.

Concerning the LOD, there was a huge difference between the two methods. The LOD of the LC-MS/MS method was 15 times better than the one of the GC-MS methods: the LOD of the LC-MS/MS method was <1 ng/mL compared to 15 ng/mL for the GC-MS method (increments by a factor of ten). Similarly, the difference in LOQ was 5 times lower in LC-MS/MS (10 ng/ml) compared to GC-MS (50 ng/mL). The LOQ for the LC-MS/MS could be set even lower than 10 ng/ml PHT (Cal 1). The FDA guidelines which claim a minimal reproducibility at the LOQ level of 20% were well below (deviation to target PHT amount: <8% in aCSF ( $n=6$ ), <4% in blood ( $n=3$ ), <9% in saliva ( $n=3$ ); accuracy: aCSF 103%, blood 101%, and saliva 106%). The LOQ of the GC-MS method and hence the lowest concentration level (Cal 1 at 50 ng/ml) of the calibration curve showed a deviation value of 19%. The LC-MS/MS method, in contrast, showed a value of only 3% deviation at the lowest Cal (10 ng/mL). This difference is of high importance, as samples with even lower concentrations could be reliably analyzed.

The calibration range (from 10 ng/mL to 2000 ng/mL) of the LC-MS/MS method was twice as large as of the GC-MS method (50 ng/mL to 1200 ng/mL). This indicates that the LC-MS/MS method is more powerful and effective over a larger range of concentration, since the linearity is given over a larger area (10 ng/mL–2000 ng/mL) compared to the GC-MS method (50 ng/mL–1200 ng/mL).

As IS, two different substances were used. MPPH as a structurally related compound was used for the GC-MS method. As IS for LC-MS/MS, deuterated PHT (PHT-D<sub>10</sub>) was used, which is the same molecule as PHT and differs only by the molecular mass (+1). All the physicochemical processes upon cleanup and analysis are identical or highly



similar for PHT and PHT-D<sub>10</sub>. MMPH, however, could be chemically affected in a different way than PHT, which could lead to a systematic bias in a given situation [15].

Regarding the sample preparation procedure, the LC-MS/MS (Figure 1) showed an important advantage compared to the GC-MS method as it only needs 3 steps of sample preparation compared to 11 steps necessary for the GC-MS method including a solid-phase extraction (SPE) and derivatization with a more critical chemical trimethylsulfonium (TMSH) (Figure 4). This resulted in significant shortening of the overall analysis: Preparation of the samples before injection for GC-MS is about ten times more time consuming than for the LC-MS/MS. For the GC-MS method, researchers needed 5 hours to prepare 25 samples (5 samples/h), whereas for the LC-MS/MS method 182 samples were prepared in 6 hours (30.3 samples/h), which corresponds to 6 times the amount of prepared samples per hour compared to the GC-MS method.

From the exposure side, the volumes are much larger and the exposure to the chemicals are more prolonged with the GC-MS method compared to the sample cleanup for the LC-MS/MS method. Especially, the derivatization agent TMSH is critical to handle because of toxicity. The risk of serious and even irreversible effects through inhalation, skin contact, or eye exposure is well known. TMSH is also considered to be teratogenic. Therefore, the potential health risk for the laboratory staff handling the samples can be reduced by the LC-MS/MS method and the elimination of a safety critical agent.

The amount of biological samples needed for the GC-MS method (50  $\mu$ L) was twice as much as for the LC-MS/MS (25  $\mu$ L). The sample volume is a critical point for PK/PD studies, where, for example, by continuing dialysis from brain in neurosurgical patients only small volumes of samples per time point/period are available. For 50  $\mu$ L dialysate about 25 minutes collecting time is necessary at the usual flow rate of  $\sim$ 2  $\mu$ L per minute [12, 16]. Therefore, not a requested specific time point, but a rather large time segment is represented which can influence the requested results. The reduced sample volume needed (25  $\mu$ L) for the LC-MS/MS analyses reduces the dialyses time needed per sample to about 15 minutes. The smaller the dialysis time, the more precise correlations of the respective tissue concentration with plasma/blood samples can be made.

Furthermore, LC-MS/MS also has the shorter run time. The time needed for 100 GC-MS analyses would be approximately 50 hours. The LC-MS/MS method, in contrast, needs only 11 hours and 40 minutes for 100 analyses. This is a time saving of more than 38 hours. While this may not be highly relevant for forensic purposes, for bedside and routine analyses (real-time) and PK/PD studies with larger numbers of samples, this factor is relevant. Also, when the time between taking a sample and the result needed is short, as it is in TDM to adjust subsequent dosing for PHT treatment, this time saving is crucial.

The costs for one way materials per sample was about 50% lower for the LC-MS/MS compared to the GC-MS method. Especially because no SPE device was needed. Also, the reduced work load for the laboratory technician must be considered as an imported cost factor.

Finally, the appropriateness of the method also depends on the biological matrix. Both methods can generally be used to measure PHT in blood and saliva, as the sample volume is less limiting. As mentioned before, however, for dialysates, the most difficult aspect is to get enough sample volume. Therefore, the LC-MS/MS method needing only half of the sample volume compared to the GC-MS method is more suited for microdialysate measurements. With respect to the LOD/LOQ, the LC-MS/MS method is also better suited for PK/PD studies, as it allows to include patients with low PHT dosages.

In addition, the newly established LC-MS/MS method was compared with a recently published LC-MS/MS method [10]. While this study measured PHT only in one body compartment (i.e., blood plasma or serum), the current LC-MS/MS method was developed and validated for the measurement of PHT in different body compartments (i.e., blood, saliva, and samples from brain tissue microdialysis often used in neurology and neurosurgery). The calibration range of the published LC-MS/MS method [10] (from 100 ng/mL to 4000 ng/mL) is appropriate for the measurement of PHT in blood plasma. As the PHT concentrations in brain tissue dialysates are much smaller than in blood plasma, the LC-MS/MS method of the current study was more appropriate for such samples, showing a lower calibration range from 10 ng/mL to 2000 ng/mL. Finally, as the aim of this study was to measure PHT in different biological matrices, a general substitute solution for blood plasma such as phosphate-buffered saline [10] could not be used. Instead, the fluid of the respective body compartment was used as calibration solution (e.g., artificial cerebrospinal fluid (aCSF) for the measurement of PHT in the brain tissue dialysates). This also eliminates a potential analytical bias due to matrix effects.

## 5. Conclusion

In this study, a LC-MS/MS method to measure PHT in different biological samples (i.e., human brain dialysate, blood, and saliva) was developed and validated under circumstances that ensured a high comparability with an established GC-MS method [8]. Overall, the study concludes that LC-MS/MS is not only better performing in human PHT concentration measuring or comparable drug PK/PD studies but is the only one to be used for bedside analysis. The time-consuming sample preparation and the long run time of the GC-MS method delay the result, which is critical in TDM. The higher sensitivity, the smaller needed sample volume, the better LOD/LOQ, the less time-consuming cleanup and sample preparation procedure, and the shorter run time make the LC-MS/MS method the preferred analytical procedure.

## Conflicts of Interest

The authors declare that they have no conflicts of interest.

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## **ARTICLE 3**

### **Free Phenytoin Assessment in Patients: Measured versus Calculated Blood Serum Levels**

Andrea Tobler, Raphael Hösli, Stefan Mühlebach, Andreas Huber

# 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  $\mu$ 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  $\leq 35$  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 %

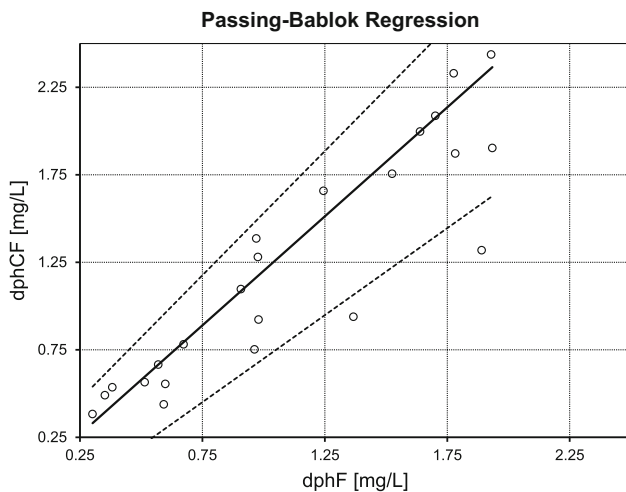
**Table 1** Total PHT levels [dphT] and measured free PHT fractions [dphF] in the investigated 23 subjects

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)		<i>t</i> Statistic
<i>Independent samples t test of the mean difference between dphF and dphCF for patients with: albumin &lt; 25 (n = 12) compared to albumin ≥ 25 (n = 11)</i>				
Mean difference = 0.13 (SD = 0.24)		Mean difference = 0.10 (SD = 1.4)		t(21) = -0.28, <i>p</i> = 0.78.

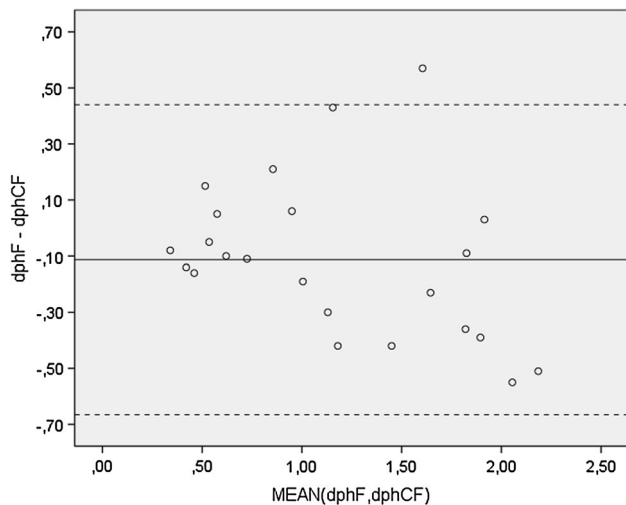
The calculated free PHT level [dphCF] was determined according to the Sheiner-Tozer algorithm. *SD* Standard deviation

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  $\pm 1.96$  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 Winkelmann 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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## 4. DISCUSSION

In a first part of this dissertation project, we have developed and validated a GC-MS method (including an appropriate clean-up method) to identify and quantitate PHT in brain microdialysate, saliva, and blood from human samples [42]. The GC-MS method is specific, sensitive, robust and well reproducible, and is therefore an appropriate candidate for the pharmacokinetic and pharmacodynamic assessment of PHT concentrations in different human biological samples. Analytical standards according to ISO 17025 and FDA guidelines have been met.

In contrast to the established EMITs which can only be applied for plasma samples (total drug concentrations), the new GC-MS method is able to determine total and free PHT concentrations in a variety of different biological samples. The GC-MS is therefore highly suited for non-routine TDM and more sophisticated pharmacological investigations such as pharmacokinetic studies in different body compartments (e.g., brain micro dialysates), correlation studies between the PHT concentration in blood plasma and the site of action, or the quantification of the free PHT fraction [41].

We were able to show that GC-MS is an analytical method which generates reliable results in different body compartments. This is an important advantage for an additional and improved TDM as almost all tissues can be addressed by the GC-MS (if appropriate samples are available) and the drug concentrations at the site of action can be correlated with the common standard plasma concentrations. The applicability of the GC-MS in different body compartments opens up new opportunities for the TDM as it allows analyzing the concentrations of an extended variety of drugs such

as antibiotics, antimycotics, aminoglycosides, antidepressants, immunosuppressants, or opiates with adapted GC-MS methods. For example, GC-MS is used in forensic toxicology to find poisons or steroids in biological samples of victims. Furthermore, in anti-doping laboratories GC-MS methods are often used to test athlete's urine samples for prohibited performance enhancing drugs (e.g., anabolic steroids) and in food analysis GC-MS detects and measures potentially harmful contaminants, spoilage, and adulteration of food [46].

The method might be of primary interest for research purposes such as toxicity, clinical trials, or pharmacokinetic and pharmacodynamic investigations of patients in critical care with brain surgery / trauma in which microdialysis is used. In neurosurgery, for example, the drugs Midazolam (as a short-acting hypnotic-sedative drug with amnestic properties), Ceftriaxon (as infection prophylaxis), Morphine (as pain killer), and antiepileptic drugs such as PHT or Levatiracetam are often administered concomitantly. For all these drugs the side of action is the brain. Thus, an adapted GC-MS method which analysis brain MD samples would allow a simultaneous TDM for all four substances.

The developed method certainly also has some limitations. The internal standard for the GC-MS method was MPPH, a structurally related compound to PHT. The fact that MPPH could be chemically affected in a different way than PHT may lead to a systematic bias. For further GC-MS method developments it would be of advantage to use deuterated molecules (e.g., in the case of PHT it would be better to use PHT-D<sub>10</sub>). Furthermore, the clean-up and extraction procedure with solid phase cartridges and 11 steps was very time consuming and costly. Thus, with regard to time and costs, further GC-MS methods could be optimized in the future.

Considering these limitations and the growing availability of LC-MS/MS methods in analyzing drugs, we have further enhanced the measurement of PHT by developing a LC-MS/MS method. The LC-MS/MS method combines the advantages of LC and MS/MS analysis. While LC has the ability to separate individual compounds from other drugs and metabolites, selective MS techniques result in superior sensitivity and specificity. We were able to validate the LC-MS/MS method according to the FDA guidelines, which are the standard when developing and validating analytical methods for new drugs in phase one. Therefore, this LC-MS/MS method would be accepted with a high probability by the FDA for investigations in humans. Thus, to ensure the real applicability of new analytical methods, it should become standard in research to use the FDA guidelines when developing and validating LC-MS/MS methods for other substances as described above (e.g., midazolam, ceftriaxone, or morphine) for TDM. Our study can thereby provide some guidance.

In order to address our objective of comparing the performance of the LC-MS/MS with the GC-MS method, we developed both methods under circumstances that ensured a high comparability. Table 1 summarizes the main advantages and disadvantages of the methods.

Regarding the sample preparation procedure, the LC-MS/MS showed an important advantage compared to the GC-MS method as it only needs 3 steps of sample preparation compared to 11 steps for the GC-MS method including a solid-phase extraction (SPE) and derivatization with the critical chemical trimethylsulfonium (TMSH). This resulted in significant shortening of the overall analysis: Preparation of the samples before injection for GC-MS is about ten times more time consuming than for the LC-MS/MS. For the GC-MS method, we needed 5 hours to prepare 25

samples (5 samples/h), whereas for the LC-MS/MS method 182 samples were prepared in 6 hours (30.3 samples/h), which corresponds to 6 times the amount of prepared samples per hour compared to the GC-MS method.

The sample volume is a critical point for TDM and PK/PD studies, where, for example, by continuing dialysis from brain in neurosurgical patients only small volumes of samples per time point / period are available. Another major problem with regard to the volume of plasma samples arises in pre-term infants with a small total blood volume, which impedes the available sample size required for lab analyses. Therefore, only a method which needs small sample volumes allows to perform a TDM for pre-term infants and other specific patient groups. The volume of biological samples needed for the GC-MS method (50  $\mu\text{L}$ ) was twice as much as for the LC-MS/MS (25  $\mu\text{L}$ ). For 50  $\mu\text{L}$  dialysate about 25 minutes collecting time is necessary at the usual flow rate of  $\sim 2$   $\mu\text{L}$  per minute. Therefore, not the specific time point, but a rather large time segment is represented which can influence the requested results. The smaller sample volume needed (we were able to measure PHT in only 5  $\mu\text{L}$  MD) for the LC-MS/MS analyses reduces the dialyses time per sample to about 15 min or even less. The shorter the dialysis time, the more frequent and therefore more precise correlations of the respective tissue concentration with plasma/blood samples can be made.

Criterion	GC-MS	LC-MS/MS
Retention time	PHT 15.12min, IS MPPH 16.15 min	PHT and PHT-D <sub>10</sub> 2.8 min
Selectivity / Sensitivity (absence of PHT)	good peak differentiation and quantification of PHT all blank samples were negative (no presence of PHT)	all blank samples were negative (no presence of PHT)
Internal standard (IS)	MPPH	100 µg/mL PHT-D <sub>10</sub>
Recovery	94.1% for QC2 100ng/mL 94.3% for QC5 1000ng/mL	89.5 % for QC1 10ng/mL 97.1% for QC3 1600ng/mL
LOD [calculated as S/N ratio of 4:1]	15ng/mL	<1ng/mL
LOQ [calculated as 5 times the response / blank]	50ng/mL	10ng/mL
Accuracy	The calibrator values showed min-max percent deviations of 1-20% for Cal 1 (50ng/mL) to Cal 6 (1200ng/mL)	The calibrator values showed min-max percent deviations of 1-8% for Cal1 (10ng/mL) to Cal8 (2000ng/mL)
Regression coefficient $r^2$	$r^2_{\text{blood}} = 0.998$ , n=2 $r^2_{\text{dialysate}} = 0.999$ , n=8 $r^2_{\text{saliva}} = 0.999$ , n=2	$r^2_{\text{blood}} = 0.996$ , n=3 $r^2_{\text{dialysate}} = 0.997$ , n=6 $r^2_{\text{saliva}} = 0.995$ , n=3
Calibration range	50-1200ng/mL	10-2000ng/mL
Run time per analysis	30 min	7 min
Injection volume of the sample	2.0 µL	10 µL
Sample preparation time	5 h for 25 samples	6 h for 182 samples
Sample preparation steps	11	3
Stability of the processed samples	Dried extracts were stable for ≥ 4 weeks (min/max deviation 4%). No effect by reinjection and storage (33h) on the auto sampler	Reinjection after 7 days showed no difference in accuracy
Sample volume needed	50 µL	25 µL

**Table 1: Comparison of the GC-MS and LC-MS/MS Method [44]**

The LC-MS/MS method is able to measure drug concentrations in different body compartments such as, for example, saliva. Small amounts of saliva (25 - 50 µL) are very easy to obtain and do not significantly affect drug concentrations in the blood. Thus, analytical methods which can analyze drug concentrations in saliva are a promising alternative to blood sample analysis for TDM.

As IS for the LC-MS/MS, deuterated PHT (PHT-D<sub>10</sub>) was used, which is the same molecule as PHT and differs only by the molecular mass (+1). All the physicochemical processes upon cleanup and analysis are identical or highly similar for PHT and PHT-D<sub>10</sub>. Thus, the potential bias when using a structurally related compound could be eliminated.

In most body compartments or tissues (e.g., saliva) or with sampling methods such as MD low drug concentrations and small sample volumes prevail. Thus, analytical methods which are able to detect low drug concentrations are preferable. Concerning the LOD, there was a huge difference between the two methods. The LOD of the LC-MS/MS method (<1 ng/mL) was 15 times better than the one of the GC-MS methods (15 ng/mL). Similarly, the difference in LOQ was 5 times lower in LC-MS/MS (10 ng/ml) compared to GC-MS (50 ng/mL). The LOQ for the LC-MS/MS could be set even lower than 10 ng/ml PHT (Cal 1). The FDA guidelines which claim a minimal reproducibility at the LOQ level of 20% were well below (deviation to target PHT amount: <8% in artificial MD (n=6), <4% in blood (n =3), <9% in saliva (n= 3); accuracy: artificial MD 103%, blood 101%, and saliva 106%). Thus, with the LC-MS/MS method also samples with low concentrations can be reliably analyzed. This is of great importance for the TDM of different substances where MD samples are used.

In general, one of the most critical points for the usefulness of an analytical method for TDM is the time needed between sampling and result. In this regard, the LC-MS/MS method shows important advantages over the GC-MS method and is the only one to be used for bedside analysis. The time needed for 100 GC-MS analyses would be approximately 50 hours. The LC-MS/MS method, in contrast, needs only 11 hours



and 40 minutes for 100 analyses. This represents a time saving of more than 38 hours. The time-consuming sample preparation and the long run time of the GC-MS method delay the result, which is critical in TDM.

Overall, the higher sensitivity, the smaller sample volume needed, the better LOD / LOQ, the less time-consuming cleaning-up and sample preparation procedure, and the shorter run time make the LC-MS/MS method the preferred analytical procedure for TDM. These advantages of LC-MS/MS methods come especially into effect when analyzing large sample batches, which is the case in pharmacokinetic, pharmacodynamic, and toxicology studies during new drug development.

In the third paper, we compared the measured free PHT concentrations with calculated free PHT concentrations using the Sheiner-Tozer formula [45]. We were able to show that, in the absence of a specific, costly, and potentially time-consuming free PHT determination, also this indirect calculation method can be useful to obtain additional information from total PHT concentration values and the albumin concentration in hypoalbuminemic patients. This study on a small group of patients could not detect any specific differences in predicting the free PHT fraction between hospitalized patients from different wards and with differing medical conditions.

The calculated (1.2 mg/L, SD = 0.7) and the measured (1.1 mg/L, SD = 0.5) free PHT concentrations significantly correlated ( $r = 0.907$ ,  $p = 0.00$ ). Furthermore, the mean differences in the low (0.10 mg/L, SD = 1.4,  $n = 11$ ) and the very low albumin group (0.13 mg/L, SD = 0.24,  $n = 12$ ) were comparable. Although the variability of the data could provide some bias, no statistically significant difference between the groups was found:  $t$  test ( $p = 0.78$ ). A linear non-parametric regression analysis based on the

Passing and Bablok procedure, which is highly suitable for method comparisons, revealed that the calculated free PHT and the measured free PHT were nearly identical.

Our findings may be extrapolated to other drugs with a high protein binding such as tacrolimus and cyclosporin (immunosuppressants) as well as vancomycin and ceftriaxon (antibiotics), or anticancer drugs such as abraxane (a protein bound paclitaxel for brain cancer treatment). For these drugs an estimated free fraction is highly useful to optimize the therapy and to perform a TDM for drug safety and successful treatment. However, as we only studied a small patient sample, research has to further investigate the utility and limitations of this theoretical approach.

Overall, for the development of a rational TDM sensitive, specific, reliable, and fast analytical methods are crucial. This dissertation shows that LC-MS/MS methods fulfill these criteria and are highly suited for analyzing drug concentrations in various matrices (body compartments). When developing a TDM an important first step is the determination of the correlation between the drug concentration at the site of action and the concentration in an easy accessible sample such as blood. The analytical method therefore has to be able to measure the drug concentrations in both body compartments. Both, the GC-MS and the LC-MS/MS method fulfilled this criterion as both measure concentrations in different body compartments and therefore allow to draw conclusions from the drug concentrations in the blood to the concentration at the site of action. Once established, these analytical methods provide reliable data which, in a next step, can be compared with less costly and simple estimation methods (e.g., Sheiner-Tozer) to calculate the free fraction of drugs based on albumin values and easy access total drug concentrations.

## 5. CONCLUSIONS

The number of drugs, which are recommended for a TDM is growing [47], including not only newly developed and designed complex drugs but also already established (critical dose) drugs [48]. With this growing demand for TDM in clinical laboratories and optimal patient care, there is a high need to develop methods with enhanced analytical performance. Based on the highly suitable reference substance and critical dose drug PHT, the present dissertation makes an important contribution in this regard by developing and validating different methods to measure or estimate drug concentrations.

The analytical methods (GC-MS, LC-MS/MS) as well as estimation methods (with mathematical algorithms) developed in this dissertation all have advantages and disadvantages for the use in TDM. The suitability of each method depends on the specific characteristics of a drug and the sample. GC-MS is, for example, highly suited for volatile and thermally stable substances in a sample and lipids in the lower mass range from 10 up to 600 Dalton. LC-MS/MS better performs for the separation of isomers, larger and non-volatile drugs, and complex peptides. Finally, estimation methods are reasonable if the correlation of the drug concentration between target tissue (free fraction) and drug concentration in blood is well established. Research needs to consider these aspects when developing new TDM methods in different situations and for different drugs. Overall, we can draw the following conclusions, which may serve as a foundation for the development of a TDM for other substances:

1. The GC-MS method is sensitive, specific, and robust and especially suited for plasma samples with relatively high drug concentrations and large sample volumes – characteristics which are often met in forensic and legal medicine. However, the method also shows major disadvantages as it is very time consuming regarding the sample preparation as well as the measurement time. Furthermore, for samples with low drug concentrations the LOD/LOQ meets not the required range and it may be that the GC-MS method cannot be extrapolated to drugs in MD samples.
2. The LC-MS/MS method was able to overcome these limitations. Compared to the GC-MS, it showed a higher sensitivity, needed a smaller sample volume, had a better LOD and LOQ, was less time-consuming in the cleaning-up and sample preparation procedure, had a shorter analysis time, and allows the simultaneous measurement of multiple drugs, which makes the LC-MS/MS method the preferred analytical procedure for (bedside) TDM and pharmacokinetic / pharmacodynamic studies. While the results are based on the model substance PHT, other promising drugs for TDM using MD samples are, for example, Midazolam, Ceftriaxon, Morphine, and Levatiracitam, which are often administered concomitantly in neurosurgery. In the future, the routinely used matrices (i.e., blood or urine) for TDM will be expanded with other sample sources like tissue biopsies, dried blood spots, and oral fluids. LC-MS/MS methods provide the advantage of drug quantification in such matrices which are easier to handle [49]. Thus, the dissertation concludes that LC-MS/MS methods are the current gold standard for TDM.

3. In the absence of the possibility to measure free PHT with analytical methods, the calculation with the Sheiner-Tozer formula represents a reasonable alternative to calculate free PHT concentrations from the total PHT and the serum albumin value of a patient to obtain an idea of the concentration levels in patients. These findings may be extrapolated to other drugs with a high protein binding such as immunosuppressants (e.g., tacrolimus and cyclosporin), antibiotics (e.g., vancomycin and ceftriaxon), or anticancer drugs (e.g., abraxane).

## 6. FUTURE RESEARCH

A better understanding of the pharmacokinetics and pharmacodynamics of a respective drug and an accurate TDM have essential benefits in clinical outcome, including quality of life of the patient. The number of drugs which are recommended for a TDM will further grow and the development of specific cost-effective and easy to use methods to measure the concentration of various drugs such as antimycotics, aminoglycosides, antidepressants, immunosuppressants as well as opioids deserves attention of researchers. When expanding the indication of already existing drugs or for new formulations of nano-drugs (e.g., Abraxane) new TDM methods can help to optimize treatments by preventing adverse drug reactions and therefore shorten hospitalizations [48]. TDM is also highly important for innovative and complex drugs and for advanced formulations using captured drugs (delivering substances specifically to target tissue in needed concentration), where the pharmacokinetics and pharmacodynamics are not yet completely investigated. An enhanced knowledge of the drug concentration at the site of action as well as the correlation between plasma drug concentration and free fraction will help to optimize drug therapy in different fields. The methods of this dissertation may serve as foundation for future studies in the following areas:

- The enhancement of TDM methods for drugs such as antibiotics to treat meningitis would be an interesting avenue for future research, as these drugs need to have a certain concentration at the specific body compartment (in this case the brain). The selection of the appropriate antibiotic therapy is difficult due to the potential of under-dosing, developing resistances, and prolonging hospitalization. Therefore, further studies may evaluate existing methods or

develop and validate new accurate methods to measure antibiotic drug concentrations in MD samples of patients suffering from meningitis.

- Substances used in anticancer therapy are often characterized by a strong relationship between dose and response, a narrow therapeutic window, and inter-individual pharmacokinetic variability. Although these drugs have characteristics which would require a TDM, today, these therapies are often applied without any accurate TDM [50]. Thus, for optimizing anticancer therapy in terms of better medical outcome due to dose adaption, future research needs to further develop the analytical methods for TDM of cancer therapies.

The oral administration of anticancer drugs (oral targeted therapies such as tyrosine kinase inhibitors) generates another complex issue in the pharmacokinetics of these drugs [51]. In addition, most of the oral targeted drugs are extremely expensive. Thus, further research to ensure that their therapeutic potential is maximized also appears justified in terms of a public health viewpoint. Future efforts should concentrate to optimize dosing of these oncology therapies.

- A further important avenue for future research is to analyze antiepileptic drugs in MD samples. We were able to demonstrate the appropriateness of LC-MS/MS methods for the analysis of concentrations of PHT as well as levetiracetam. By comparing the concentrations in MD samples with the blood concentration a better understanding of the pharmacokinetic and pharmacodynamics of these drugs in critically ill patients would be possible and the therapy could be individualized.

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## 8. LIST OF PUBLICATIONS AND PRESENTATIONS

### 8.1 Peer Reviewed Articles

1. Hösli R, König S, Mühlebach S. Development and Validation of an LC-MS/MS Method and Comparison with a GC-MS Method to Measure Phenytoin in Human Brain Dialysate, Blood, and Saliva. *J Anal Methods Chem.* April 2018.
2. Tobler A, Hösli R, Mühlebach S, Huber A. Free Phenytoin Assessment in Patients: Measured versus Calculated Blood Serum Levels. *Int J Clin Pharm* 2016; 38(2): 303-9.
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4. Annaheim B, Wangmo T, Belardi A, Meyer A H, Hoesli R, Bretschneider W, Handtke V, Lutters M, Elger B S. Polypharmacy and Drug-Drug Interactions among Younger and Older Male Prisoners in Switzerland. *International Journal of Prisoner Health: Under Review*

## 8.2 Poster Presentations

1. Determination of Free Phenytoin Blood Concentrations in Patients: Measured Versus Calculated Serum Levels  
Hösli R, Tobler A, Mühlebach S.  
Swiss Pharma Science Day 2014, Swiss Pharma 36 (2014), Poster Nr. P-19
2. Comparison of LC-MS and GC-MS with Internal Standards to Analyze Phenytoin from Different Biological Samples  
Hösli R, König S, Mühlebach S  
Swiss Pharma Science Day 2011, Swiss Pharma 33 (2011), Poster Nr. P-35
3. LC-MS versus GC-MS in the Analysis of Phenytoin in Patients' Samples: Evaluation Criteria and a Scoring Approach.  
Hösli R, König S, Mühlebach S  
1. Schweizer Apothekerkongress, 30.11.-1.12.2011, Interlaken Schweiz,  
Poster Nr. PP56
4. Determination of Phenytoin from Human Brain Microdialysis and other Biological Samples: Development and Validation of a GC-MS Method  
Hösli R, Tobler A, Aeby B, Landolt H, Mühlebach S.  
FIP Pharmaceutical Sciences World congress 2010 / AAPS Annual Meeting and Exposition 14.-18.11.2010, New Orleans USA, Poster Nr. R6363
5. A Validated Phenytoin Analysis from CNS ex vivo Brain Microdialysis Samples, Blood and Saliva by GC/MS



Hösli R, Tobler A, Aeby B, Landolt H, Mühlebach S.

Swiss Pharma Science Day 2009, Swiss Pharma 31 (2009) Nr. 10, Poster Nr.

P-40

6. Lagerung von Medikamenten in Lebensmittelkühlschränken: Eine günstige Alternative oder ein Sicherheitsrisiko?

Grunert S, Zysset T, Hösli R

Jubiläumskongress der Gesellschaft Schweizerischer Amts- und Spitalapotheker 20-21.11.2008, KKL Luzern, Schweiz

7. Phenytoin Analysis of CNS Microdialysis Samples from Neurosurgical Patients: Definition of a GC-MS Method and its Validation

Hösli R, Tobler A, Aeby B, Mühlebach S.

Jubiläumskongress der Gesellschaft Schweizerischer Amts- und Spitalapotheker, 20.-21.11.2008, KKL Luzern, Schweiz.

### 8.3 Oral Presentations

Project Layout, University Hospital of Lausanne. Lausanne CHUV 2012:

Prospective Study of Phenytoin and Keppra in ICU Patients.

FPH-Prüfungsvortrag, Universität Bern. Bern, November 2011. A Quantitative Phenytoin GC-MS Method for samples from Human ex Situ Brain Microdialysis, Blood and Saliva Using Solid Phase Extraction and its Validation.

Journal Club, Institut für Rechtsmedizin, Universität Bern. Bern, 2010 Phenytoin Analyse mittels LC-MS/MS.

Projekt-Vorstellung, Universität Basel. Basel 20. Januar 2009 Entwicklung einer GC/MS-Methode zur Phenytoin-Bestimmung im ZNS-Dialysat.

Journal Club, Institut für Rechtsmedizin, Universität Bern. Bern, April 16, 2008. Bestimmung und Pharmakokinetik von Medikamenten im ZNS: Ein Beitrag zur Optimierung der Arzneimitteltherapie.

BFB - Bildung Formation Biel-Bienne. Biel GKT 2. Lehrjahr Pharmaassistentinnen 2010-2014: Thema Augen.

BFB - Bildung Formation Biel-Bienne. Biel GKT 2. Lehrjahr Pharmaassistentinnen 2009-2014: Thema Bewegungsapparat.

## 8.4 Congress Participations

12th Congress of the EAHP 2007, 21.-23.3.2007, Bordeaux, France

30<sup>th</sup> ESPEN Congress 2008, 13.-16.9.2008, Florence, Italy

Jubiläumskongress der Gesellschaft Schweizerischer Amts- und Spitalapotheker GSASA, 20. - 21.11.2008, Kultur- und Kongresszentrum Luzern, Schweiz

Swiss Pharma Science Day 2009, 2.9.2009, Bern, Schweiz

12 ISOPP Symposium of 6th international society of oncology Pharmacy Practitioners,  
5.-8.5.2010, Prague, Czech Republic

13<sup>èmes</sup> Journées du Gerpac, 6.-8.10.2010, Presqu'île de Giens, France

FIP Pharmaceutical Sciences World congress 2010 / AAPS Annual Meeting and Exposition, 14.-18.11.2010, New Orleans, USA

Swiss Pharma Science Day 2011, 31.8.2011, Bern, Schweiz

14<sup>èmes</sup> Journées du Gerpac 5.-7.10.2011, Presqu'île de Giens, France

15<sup>èmes</sup> Journées du Gerpac 3.-5.10. 2012, Presqu'île du Ponant, France

18th congress of the EAHP, 13.-15.3.2013, Paris, France

16<sup>èmes</sup> Journées du Gerpac, 2.-4.10.2013, Presqu'île de Giens, France

19th congress of the EAHP, 26.-28.3.2014, Barcelona, Spain

Swiss Pharma Science Day 2014, 20.8.2014, Bern, Schweiz

17èmes Journées du Gerpac, 1.-3.10.2014, Presqu'île de Giens, France

18èmes Journées du Gerpac, 7.-9.10. 2015, Presqu'île de Giens, France

Gsasa Kongress 2015, 26-27.11.2015, Zürich, Schweiz

21th congress of the EAHP, 16.-18.3.2016, Vienna, Austria

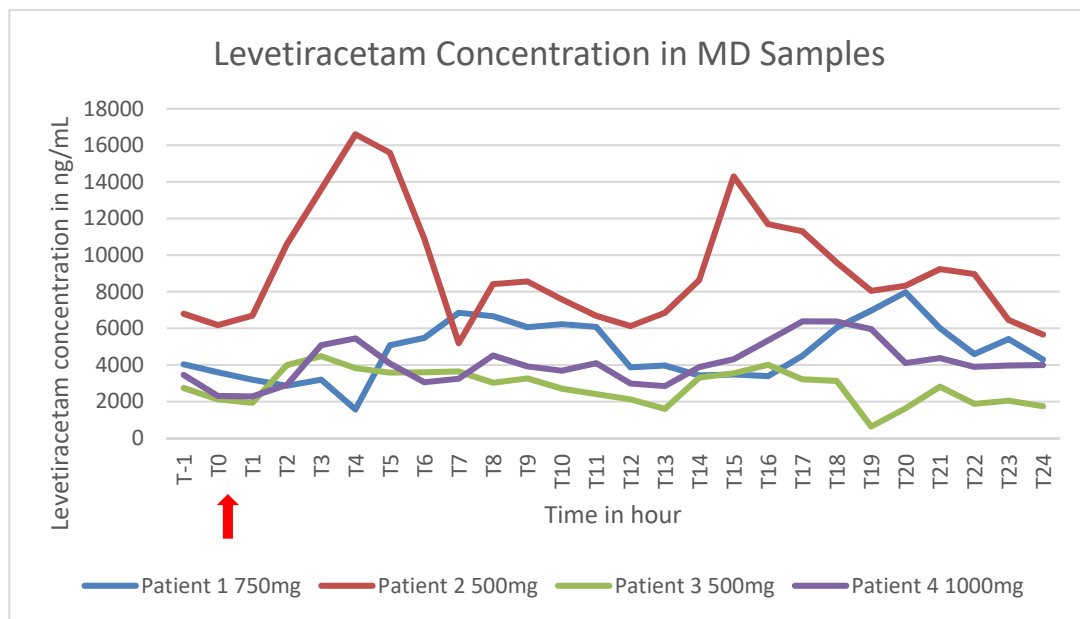
19èmes Journées du Gerpac 5.-7.10. 2016 Presqu'île de Giens, France

20èmes Journées du Gerpac 4.-6.10. 2017 Presqu'île de Giens, France

23th congress of the EAHP, 21.-23.3.2018 Gothenburg, Sweden

## 9. Appendix

## Appendix A: Measurement of Levetiracetam Concentrations



Measurement of levetiracetam concentrations in MD samples of 4 Patients with cranial trauma. The patients received different dosages of levetiracetam at time  $T_0$ .

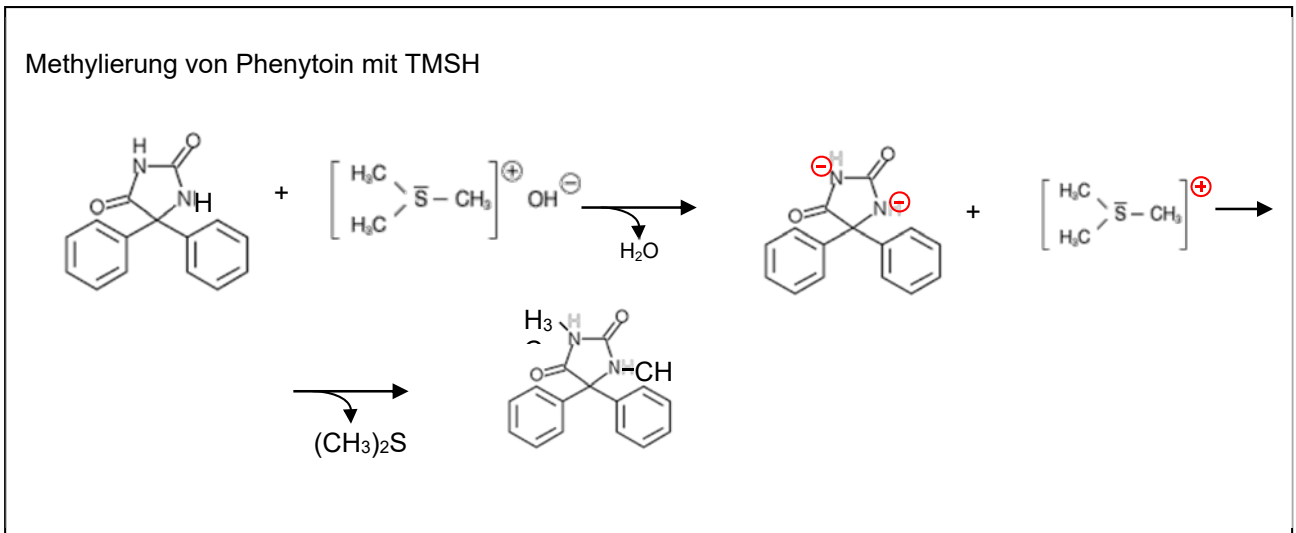
## Appendix B: Method SOPs

### 1. Methoden SOP zur Bestimmung von Phenytoin mittels GC-NPD-MS (IRM Bern)

Bestimmung von freiem Phenytoin im ZNS-Mikrodialysat / Liquor mittels GC-MS-NPD (Inv. 283). Die SOP wurde durch A. Tobler und R. Hösli erarbeitet. Da die Analysen am IRM in Bern durchgeführt wurden, wurde die SOP dem Qualitätsmanagement des IRM's angepasst. Dies geschah mit der Unterstützung von Martine Gasser.

#### Zweck, Prinzip und Bedeutung

Die vorliegende Methode beschreibt die quantitative und qualitative Bestimmung von freiem Phenytoin im Mikrodialysat (ZNS, Liquor). Die Probenvorbereitung besteht in der Zugabe eines Internen Standards. Anschliessend wird die vorbereitete Probe mittels Festphasen-Extraktion (SPE) gereinigt. Der Extrakt wird derivatisiert und mit GC/MSD-NPD analysiert.



[Eigene Darstellung nach: Trimethylsulfonium Hydroxide: A New Methylating Agent; Kiyoshi Yamauchi, Toshizumi Tanabe, Masayoshi Kinoshita; J. Org. Chem., Vol. 44, No. 4, 1979 und Selective 2-O-Methylation of Pyrimidine-Ribonucleosides by Trimethylsulfonium Hydroxide in the Presence of  $Mg^{2+}$  and  $Ca^{2+}$  Ions; Kiyoshi Yamauchi, Toshizumi Tanabe, Masayoshi Kinoshita; Bull. Chem. Soc. Jap; Vol. 59; 2947-2949; 1986]

Plasmakonzentrationen von Phenytoin:

Phenytoin	Therapeutischer Bereich [ng/mL]	Toxischer Bereich [ng/mL]
Gesamt-Phenytoin (Plasma)	10'000-20'000	>20'000
Freies Phenytoin (Plasma bzw. Mikrodialysat)	600-2'400	>2'400

#### Geltungsbereich

Die Bestimmung von freiem Phenytoin im Mikrodialysat (ZNS, Liquor) wird im Rahmen der Dissertationen von Frau Andrea Tobler und Herrn Raphael Hösli durchgeführt. Diese Analysenmethode ist somit für das IRM nicht bindend, kann aber vom IRM verwendet werden.

## Begriffe und Abkürzungen (alphabetisch)

aCSF	artificial cerebrospinal fluid (künstliche Hirnflüssigkeit)
BW(-Dialysat)	Blindwert(-Dialysat)
HAc	Essigsäure
ISTD	Interner Standard (hier: MPPH)
Kal. X	Kalibrator x
LOD	Limit of detection (Detektionsgrenze)
LOQ	Limit of quantification (Bestimmungsgrenze)
MPPH	5-(p-Methylphenyl)-5-Phenylhydantoin
GC- MS-NPD	Gaschromatograph mit Massenselektivem und Stickstoff-Phosphor-Detektor
P	Probe(n)
PDL	Phenhydantolösung
PHT	Phenytoin
PhtL	Phenytoinlösung
Px-A	Probenbezeichnung des Biosamples x der Serie A
Px-B	Probenbezeichnung des Biosamples x der Serie B
QC	quality control, Qualitätskontrolle
QCx-A	Probenbezeichnung der Qualitätskontrolle x der Serie A
QCx-B	Probenbezeichnung der Qualitätskontrolle x der Serie B
RG	Reagenzglas
RW	Richtwert
SPE	Solid phase extraction, Festphasenextraktion
SST	System suitability test
TMSH	Trimethylsulfoniumhydroxid (Derivatisierungsmittel)
ZNS	Zentrales Nervensystem

## Benötigte Materialien und Chemikalien

Glaswaren, Geräte und weitere Ausrüstung

### *Glaswaren*

Messzylinder 50 mL

Messkolben 10 mL mit Deckel

Messkolben 20 mL mit Deckel

Messkolben 50 mL mit Deckel

Messkolben 500 mL mit Deckel

Messkolben 1000 ml mit Deckel

Glas-RG mit Schraubdeckel

GC-Vials mit Alu-Caps

Diverse Glaswaren zum Aufbewahren der hergestellten Lösungen (Schott-, Pyrex-, Duran-Flaschen)

### *Pipetten*

Pasteur-Pipetten

Diverse Micropipetten:

Socorex 2-20 µL, 20-200 µL, 100-1000 µL

Gilson bis 10 µL, bis 25 µL

Diverse Glasvollpipetten

### *Geräte*

GC-MS-NPD (Inv. 283)

Software HP Chem Station

GC-Säule:

Vacmaster, Vac Elut SPS 24CK (Inv. 2417), Varian

Vortex Genie 2 (z.B. Inv. 740)

Abdampfblock (Inv. 2361), witec ag

Oberschalenwaage (Inv. 2420), Mettler Toledo



### *Sonstiges*

Extraktionssäulen, Bond Elut® Solid Phase Extraction LRC-Certify, 130MG, Art. Nr. 12113050, Varian

Capper, Decapper

Sofern nicht ausdrücklich ausgeschlossen können vergleichbare Glaswaren und Geräte verwendet werden.

### **Chemikalien und Reagenzien**

Methanol z.A., Hersteller Merck, Art. Nr.: 1.06009.1000

Aceton z.A., Hersteller Merck, Art. Nr. 1.00014.1000

Essigsäure 100% (Eisessig) z.A., Hersteller Merck, Art. Nr.: 1.00063.1000

Reinstwasser Typ I

pH-Indikatorstäbchen 0-14, Hersteller Merck, Art. Nr.: 1.09535.0001

SPE-Extraktionssäulen Bond Elut Certify 130 mg LRC, Hersteller Varian

Stickstoff (N<sub>2</sub>), Hersteller Carbagas

Helium, Hersteller Carbagas

Citronensäure Monohydrat, Ph. Eur. /USP, ADM Ringaskiddy Ireland

Natriumhydroxid in Plätzchen, Hersteller Merck, Art. Nr. 1.06498.1000

Magnesiumchlorid-Hexahydrat z.A., Hersteller Merck, Art. Nr. 1.05833.0250

Calciumchlorid z.A., Hersteller Merck, Art. Nr. 2083

Natriumchlorid, Hersteller Merck, Art. Nr. 6404

Kaliumchlorid z.A., Hersteller Merck, Art. Nr. 1.04936.0500

Trimethylsulfoniumhydroxid (TMSH) 0.2 M in Methanol, Hersteller Macherey-Nagel AG, Art. Nr. 701520.110, Schweiz

0.2 M NaOH (aus Natriumhydroxid in Plätzchen, Hersteller Merck, Art. Nr. 1.06498.1000)

1 M HCl, IRM

0,01 M HAc (aus Essigsäure 100% (Eisessig) z.A., Hersteller Merck, Art. Nr.: 1.00063.1000)

Sofern nicht ausdrücklich ausgeschlossen, können vergleichbare Chemikalien und Reagenzien verwendet werden.

### **Referenzsubstanzen**

Phenytoin, Referenzsubstanz, Desitin Arzneimittel GmbH, Desitin Pharma GmbH, Liestal, Schweiz

Phenytoin, CRS, Council of Europe, European Pharmacopoeia, Strasbourg

5,5-Diphenylhydantoin purum ≥96%, Fluka Chemika, Art. Nr. 43070

Phenhydan, Injektionslösung 250 mg Phenytoin/5 mL, Ampullen, Desitin Pharma GmbH, Liestal, Schweiz

Sofern nicht ausdrücklich ausgeschlossen, können Referenzsubstanzen gleicher Qualität anderer Hersteller verwendet werden.

Interner Standard

5-(p-Methylphenyl)-5-Phenylhydantoin >99%, Sigma-Aldrich Chemie GmbH, Steinheim, Deutschland

### **Referenzdialysate**

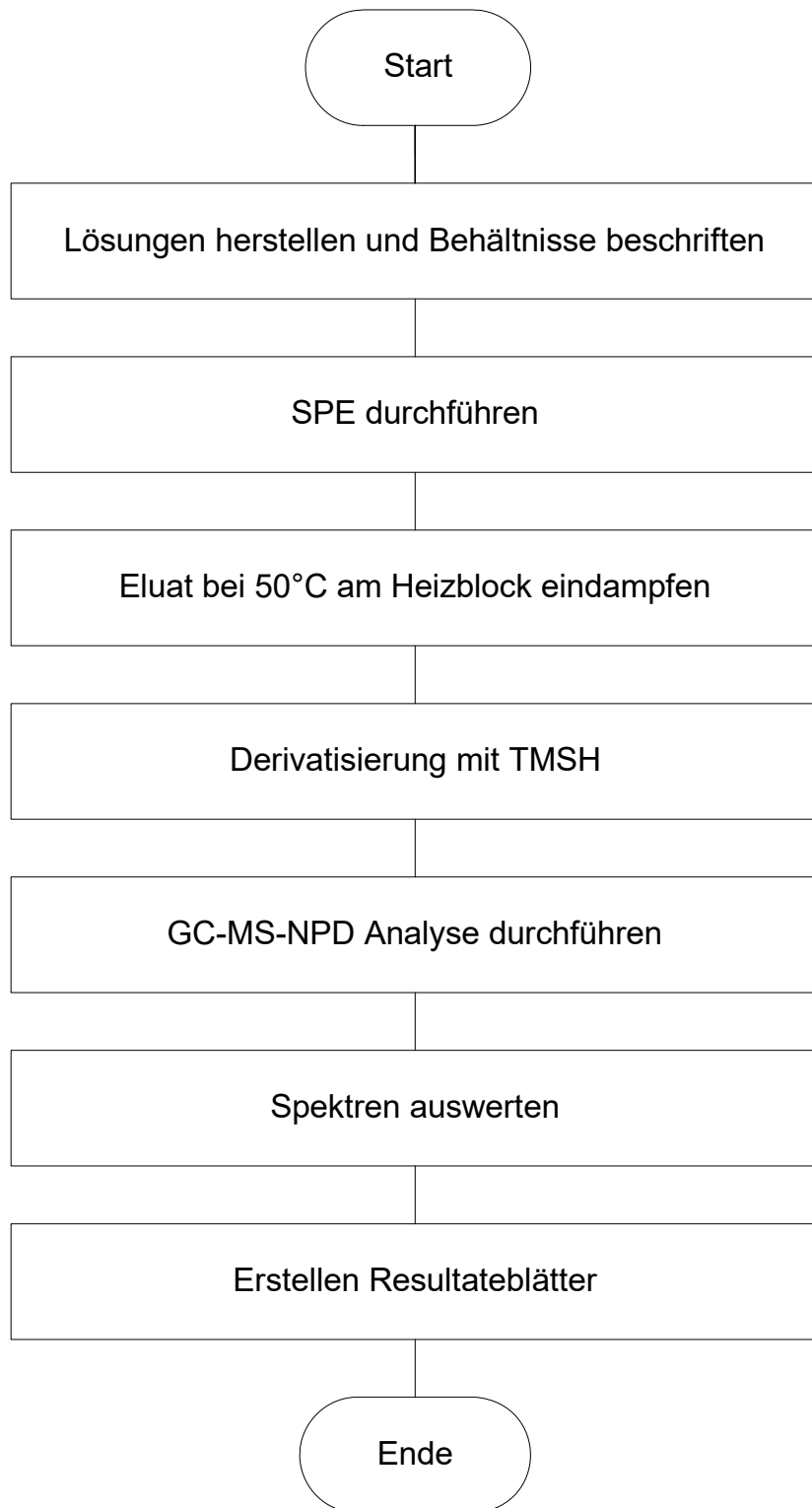
Es wird selber hergestellte, künstliche Hirnflüssigkeit (aCSF) verwendet. Herstellung sieht unter Kapitel 0.

### **Blindwerte**

Zurzeit sind keine vorhanden. Es ist geplant, Blindwerte von der Neurochirurgie Aarau zu bekommen. Falls dies nicht möglich sein sollte, wird NaCl 0,9% als Blindwert verwendet.

## Vorgehen

### Flussdiagramm



## Kalibration

Die Kalibration wird automatisch durch HP Chem Station berechnet, sofern die Kalibratoren Kal.1-Kal.6 auch als solche im Programm eingetragen sind. Die Kalibratoren werden aus den Kalibrationslösungen hergestellt, wie unter Kapitel 0 beschrieben.

## Lösungen

### Arbeitslösungen

Citrat-Pufferlösung pH 5.0 R:

20.1 g Citronensäure und 8.0 g Natriumhydroxid ad 1000 mL H<sub>2</sub>O; gut mischen, bis zur vollständigen Auflösung. pH-Wert mit pH-Stäbchen kontrollieren. (Wenn nötig, pH-Wert mit verdünnter Salzsäure einstellen). Die Pufferlösung wird im Kühlschrank bei 2-8°C gelagert und ist ca. 6 Monate haltbar.

[4010700 nach Ph. Eur. 5. Ausgabe, Grundwerk 2005]

Künstliche Hirnflüssigkeit (aCSF):

NaCl, KCl, CaCl<sub>2</sub> und MgCl<sub>2</sub> ad 1000 mL H<sub>2</sub>O, gut mischen, bis zur vollständigen Auflösung. Der pH-Wert dieser Lösung beträgt 7.4 (Wenn nötig, pH-Wert mit 0.2M NaOH einstellen). Die künstliche Hirnflüssigkeit wird im Kühlschrank bei 2-8°C gelagert und ist ca. 6 Monate haltbar.

Zusammensetzung nach: Firma CMA, Stockholm, Schweden

Elektrolyt(e)	Massengewicht* M [g/mol]	Konzentration [mmol/l]	Konzentration [mol/l]	Berechnete Menge [g] (Konz.[mol/l] x M [g/mol])
NaCl	58,4425	147	0,147	8,5910
KCl	74,5510	2,7	0,0027	0,2013
CaCl <sub>2</sub>	110,9834	1,2	0,0012	0,1332
MgCl <sub>2</sub> x6H <sub>2</sub> O	203,3022	0,85	0,00085	0,1728

\* nach Angaben des Periodensystems der Elemente von Macherey-Nagel [www.mn-net.com]

### Kalibrationslösungen

#### Phenytoin-Lösungen:

Phenytoin-Stammlösung:

0,050g der Referenzsubstanz wird mit Methanol ad 50 mL aufgelöst. Als Referenzsubstanz kann entweder Phenytoin Desitin, Phenytoin CRS oder 5,5-Diphenylhydantoin verwendet werden, gut mischen, bis zur vollständigen Auflösung.

⇒ 1 mg/mL (1 ng/μL) in Methanol

Diese Lösungen werden 1:10 mit MeOH weiterverdünnt: 100 μg/mL (100 ng/μL) in MeOH

⇒ PhtL ①

PhtL ① wird 1:10 mit MeOH weiterverdünnt: 10 μg/mL (10 ng/μL) in MeOH

⇒ PhtL ②

PhtL ② wird 1:10 mit MeOH weiterverdünnt: 1 μg/mL (1 ng/μL) in MeOH

⇒ PhtL ③

Die Phenytoin-Stammlösung wird im Kühlschrank bei 2-8°C gelagert und ist ca. 12 Monate haltbar.

Diese Lösungen werden für die Herstellung der Kalibratoren verwendet.

#### Phenhydan-Lösungen:

Phenhydan-Stammlösung:

Die Stammlösung kann auch aus Phenhydan hergestellt werden. Dazu 1 mL Phenhydan® mit Methanol ad 50mL versehen, gut mischen, bis zur vollständigen Durchmischung

⇒ PDL 1 mg/mL (1 ng/µL) in Methanol

Diese Phenhydan-Lösung wird 1:10 mit MeOH weiterverdünnt: 100 µg/mL (100 ng/µL) in MeOH

⇒ PDL ①

PDL ① wird 1:10 mit MeOH weiterverdünnt: 10 µg/mL (10 ng/µL) in MeOH

⇒ PDL ②

PDL ② wird 1:10 mit MeOH weiterverdünnt: 1 µg/mL (1 ng/µL) in MeOH

⇒ PDL ③

Die Stammlösung PDL wird im Kühlschrank bei 2-8°C gelagert und ist ca. 12 Monate haltbar. Diese Lösungen werden für die Herstellung der QC verwendet.

### Interner Standard (ISTD)

0.050 g 5-(p-Methylphenyl)-5-Phenylhydantoin ad 50 mL Methanol, gut mischen, bis zur vollständigen Auflösung (Ultraschallbad).

⇒ ISTD 1 mg/mL (1 ng/µL) in Methanol

Diese Lösung (ISTD) wird 1:10 mit MeOH weiterverdünnt: 100 µg/mL (100 ng/µL) in MeOH

⇒ ISTD ①

Die Lösung ISTD wird im Kühlschrank bei 2-8°C gelagert und ist ca. 12 Monate haltbar.

### Vorbereitung des SST

Der SST dient zum Testen der Methode. Der SST muss vor jeder Serie neu durchgeführt und gemessen werden. Die Lösungen werden jeweils frisch hergestellt.

- 100 µL PhtL ③ in ein GC-Vial pipettieren
- 24 µL ISTD ① dazugeben
- Vortexen
- Eindampfen, N<sub>2</sub>, 50°C
- 100 µL TMSH dazugeben
- verschliessen, gut vortexen (Diese Lösung entspricht dem Kalibrator 1).

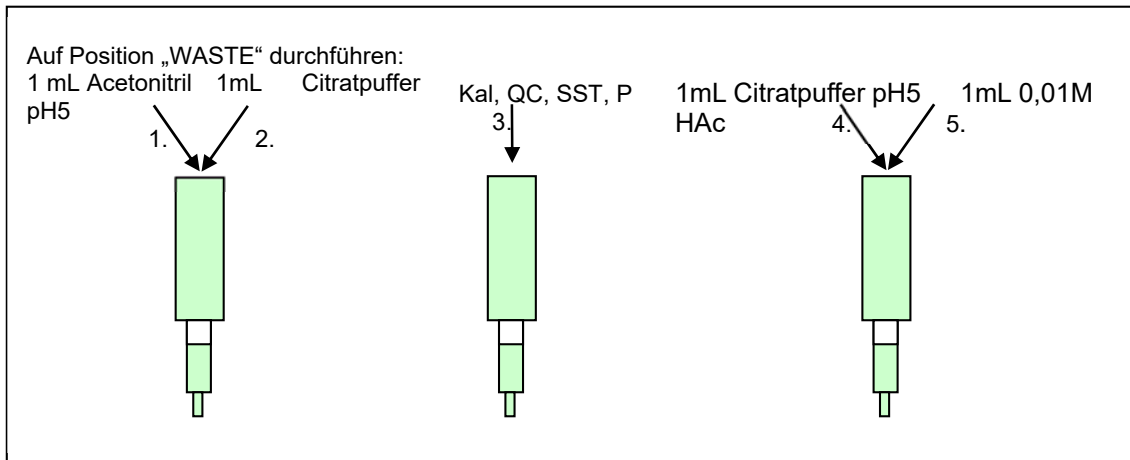
Die Bestimmung von Proben (P) und Qualitätskontrollen (QC) erfolgt doppelt.

Besondere Massnahmen: Alle Lösungen auf Raumtemperatur erwärmen lassen. 1 mL Dialysat oder aCSF in Pyrex-RG pipettieren. Ansätze gemäss Ansatzschema zupipettieren und kurz vortexen:

Probe	Phenytoin [ng]	ISTD [ng]	Zusätze / 1 mL Dialysat oder aCSF
Kal. 1	50	1200	50 µL PhtL ③ + 12 µL ISTD ①
Kal. 2	150	1200	15 µL PhtL ② + 12 µL ISTD ①
Kal. 3	300	1200	30 µL PhtL ② + 12 µL ISTD ①
Kal. 4	600	1200	60 µL PhtL ② + 12 µL ISTD ①
Kal. 5	1000	1200	10 µL PhtL ① + 12 µL ISTD ①
Kal. 6	1200	1200	12 µL PhtL ① + 12 µL ISTD ①
Kontrolldialysat: aCSF und SST	1200	1200	100 µL PhtL ③ + 24 µL ISTD ①
QC 1	100	1200	10 µL PDL ② + 12 µL ISTD ①
QC 2	1000	1200	10 µL PDL ① + 12 µL ISTD ①
BW-Dialysat	---	1200	+ 12 µL ISTD ①
Proben (Dialysat)	---	1200	+ 12 µL ISTD ①

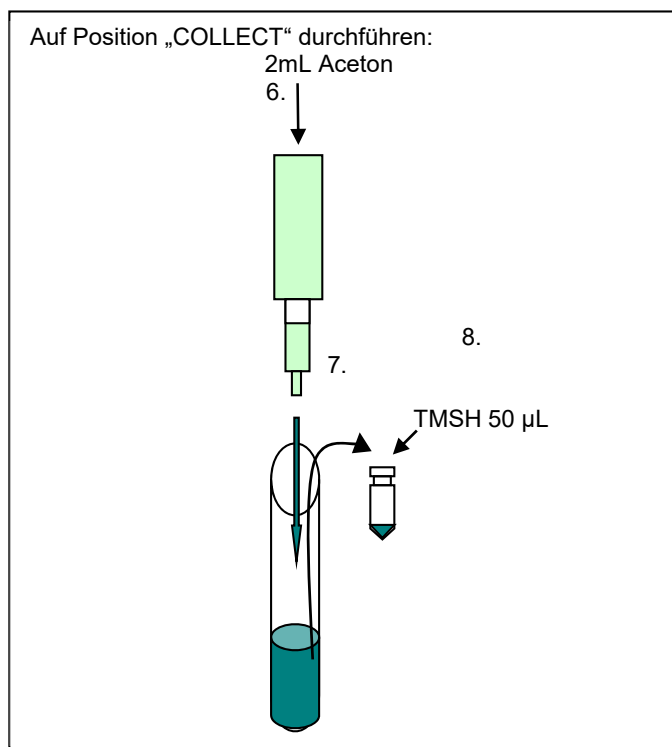
## Festphasenextraktion (SPE) und Derivatisierung

SPE:



Säulen (Bond Elut Certify 130 mg, LRC) vorgängig beschriften:

1. Konditionieren mit 1 mL Acetonitril (ohne Vakuum)
2. Äquilibrieren mit 1 mL Citrat-Puffer pH 5 (ohne Vakuum)  
nicht trocken laufen lassen
3. Probenaufnahme: Gesamtes Volumen (mit Pasteur-Pipette), (ohne Vakuum)
4. Säulen waschen mit 1 mL Citrat-Puffer pH 5 (mit schwachem Vakuum)
5. 1 mL HAc 0.01M (mit schwachem Vakuum). Ca. 5 min trockensaugen, bei vollem Vakuum



6. Eluieren mit 2 mL Aceton (ohne Vakuum)  
Ohne Vakuum eluieren lassen, in Glas-RGs auffangen, am Schluss ca. 10 s Vakuum anlegen
7. Ganzes Eluat (mit Pasteur-Pipette) in GC-Vial überführen und bei 50°C mit N<sub>2</sub> bis zur Trockene eindampfen
8. Zum Derivatisieren den Rückstand mit 50 µL TMSH aufnehmen  
GC-Vial mit Alufolie und Alu-Cap verschliessen und gut vortexen

### Instrumentelle Bestimmung

Die Analyse des derivatisierten Extraktes erfolgt auf dem GC-MS-NPD (Inv. 283).

Spüllösung: Methanol

Gerätetune: atune

Zur Inbetriebnahme des NPD wird, nachdem die GC-Methode geladen wurde und die Statusanzeige nicht mehr NOT READY anzeigt, der Einstellknopf gedreht. Der schwarze Riegel unter dem Knopf muss zum Drehen nach links gedrückt werden und zum Feststellen des Knopfes wieder nach rechts gedrückt werden. Der Knopf wird für höhere Grundwerte im Uhrzeigersinn und für tiefere Grundwerte oder zum Ausschalten im Gegenuhrzeigersinn gedreht. Es wird solange im Uhrzeigersinn gedreht bis das NPD-Signal (GC-Display Signal A) bei der gewählten Methode ca. 40 (Signal sollte zwischen 30 und 50 Einheiten liegen) Einheiten anzeigt. Der NPD muss anschliessend während 15 Minuten stabilisieren, ansonsten bleibt die Basislinie nicht konstant. Den eingestellten Wert (ca. 40 Einheiten) nochmals kontrollieren und gegebenenfalls nachregeln. Dies ist der Wert des Grundrauschens.

Wenn die Probe eingespritzt wurde und den NPD erreicht, kann es für ein paar Sekunden zu Schwankungen kommen, das NPD Signal erholt sich dann wieder.

### Akquisition

Die folgenden Methoden stehen zur Verfügung:

GC-MS-Methode (Scan-Methode): PHT.M (RH06.M )

Sequenz: PHT.S (für qualitative und quantitative Analyse, für Methodentest SST)

Die eingescannte GC-Methode befindet sich im PIOS.

Massen der Fragmente, Definition der Qualifier

Analyt

Interner Standard

Phenytoin: **280**, 203, 194, 118  
203, 194, 118

5-(p-Methylphenyl)-5-Phenylhydantoin: **294**,

### Methodentest (SST)

Das Gerät muss vor jeder Messserie mit dem SST getestet werden. Dieser Test soll sicherstellen, dass das System für die Analyse bereit ist. Zu diesem Zweck wird ein SST (Kal. 1 = Level 1) direkt vor der Kalibration und vor den Proben P gemessen (Herstellung siehe Kap. 5.3.1.3). Mit Hilfe des SST wird die Empfindlichkeit optimiert und Retentionszeiten sowie allfällige Interferenzen und die Chromatographie überprüft.

## Durchführung des SST

Aktualisierung der SCAN-Methode:

- **"Level 1"** im SCAN-Modus mit der Methode PHT.M (RH06.M) messen zur Überprüfung der Retentionszeiten
- wenn nötig die SCAN-Zeitfenster in der SCAN-Methode anpassen

Optimierung der Empfindlichkeit:

- **"Level 1"** mit der SCAN-Methode messen. Die Signalstärke muss mindestens eine Intensität von 100'000 haben.

Kontrolle der Messungen mit der SCAN-Methode:

- **"Level 1"** im SCAN-Mode mit der aktualisierten SCAN-Methode messen
- Auswerten mit der Auswertungsmethode  
Berechnet wird mit der Kalibration der letzten Serie.

## Beurteilung des SST

Kriterien für die Beurteilung der Testlösung:

- Phenytoin wird erkannt und berechnet
- ISTD wird erkannt und berechnet
- Die Peaks weisen kein starkes Tailing auf
- Die Empfindlichkeit des Systems ist ausreichend
- Die Schwankungen der errechneten Werte an Phenytoin im Bezug auf 1. und 2. Einspritzung (vor Kalibration und vor Probenserie) sind gering

Werden ein oder mehrere Kriterien nicht erfüllt, muss über das weitere Vorgehen entschieden werden.

## Kontrollen

### *Blindprobe (BW-Dialysat)*

Eine Blindprobe wird bei jeder Probenserie extrahiert und analysiert. Die Probe darf keine Resultate im Bereich des PHT-Peaks aufweisen. Weiter sind keine Interaktionen der Inhaltsstoffe mit PHT zulässig, da diese die Resultate verfälschen könnten.

### *Referenz-Dialysat (aCSF)*

Als Referenz-Dialysat wird selber hergestellte, gespikte aCSF verwendet. Dieses wird bei jeder Serie im Doppel extrahiert und einfach analysiert (Doppelbestimmung). Die gespikten Referenz-Dialysate werden gleichmässig über die Sequenz verteilt.

Bezeichnung	Soll [ng/ml]	PDL ① [µL]	PDL ② [µL]	ISTD ① [µL]	aCSF [µL]
QC1-A; QC1-B	100		10	12	1000
QC2-A; QC2-B	1000	10		12	1000

*Kriterien für die gespikten Referenz-Dialysate:*

- Phenytoin wird erkannt und berechnet
- Abweichung < 10%                      Sequenz valide
- Abweichung >10% und < 30%      Sequenz valide
- Abweichung > 30%                      Sequenz nicht valide

$[(\text{Resultat QC1-A} + \text{Resultat QC1-B})/2] - \text{Soll} \rightarrow \text{Abweichung in \% für QC1 resp. QC2 etc.}$

Die % der QC1-QC5 werden addiert und dann durch 5 dividiert  $\rightarrow$  Gesamtabweichung in %  
*SST direkt*

Der SST wird mitgeführt, um die Recovery und mögliche Veränderungen während der Serie zu überprüfen. Sämtliche Auswertungen der Kontrollproben und des SST werden im Serienordner abgelegt (siehe auch Kapitel 0 ff.).

## **Auswertung**

Die qualitative und quantitative (SCAN) Auswertung erfolgt mit der Auswertungsmethode PHT.M (RH06.M). Wenn nötig, müssen zuerst die Retentionszeiten in der Auswertungsmethode (Calibrate/Edit Compounds) angepasst werden. Ist die Integration nicht zufriedenstellend, müssen die entsprechenden Integrationsparameter optimiert und abgespeichert werden.

### Qualitative Auswertung

Zur Identifizierung von Phenytoin wird ein Chromatogramm (SCAN) geladen und die Massenspektren der jeweiligen Peaks mit den Spektren der Datenbank verglichen. Phenytoin ist eindeutig identifiziert, wenn Retentionszeit und Massenspektrum übereinstimmen. Hierbei dient der SST zur genauen Bestimmung der Retentionszeit. Die Chromatogramme werden auch auf das Vorhandensein anderer Metaboliten überprüft, Hinweise werden auf dem Resultateblatt vermerkt.

### Beurteilung der Chromatogramme:

Positiv

Phenytoin ist sichtbar

Massenspektrum und Retentionszeit sind korrekt

Peak ist > dreimal Rauschen

Hinweis in Spuren

Phenytoin ist sichtbar

Massenspektrum und Retentionszeit sind korrekt

Peak ist ein- bis dreimal Rauschen

Negativ

Kein Peak ist sichtbar

Spektrenbibliotheken:

Direkt über Beat Aebi d.h. el. Übertragung (offline)

Für die Auswertung von full-scan Spektren sind diese Bibliotheken und Werte zu verwenden. Zu Testzwecken können auch andere Bibliotheken verwendet werden. Es ist aber wichtig, dass die Daten vor der Bibliothekssuche überprüft und auch so in der Methode gespeichert werden.

### Quantitative Auswertung

Berechnung

Die Berechnung der Kalibration erfolgt automatisch durch die Chemstation-Software. Es handelt sich um eine lineare Regression mit ISTD. In der Regel erfolgt die Berechnung mit Hilfe der MS-Resultate.

Übertragung der Flächen (Kalibration)

Ausgewertet wird mit Hilfe des MS. Die Übertragung der Flächen wird anhand des mitgeführten Referenz-Dialysates beurteilt. Die Übertragung der Flächen wird mit einer 1-Punkt-Kalibrierung mittels Interner Standard-Methode gemacht, indem die Response des



ISTD für die zu bestimmenden Substanzen übernommen werden. Die Signale werden mittels NPD kontrolliert. Die Konzentration des ISTD in der Probe beträgt je 1200 ng/mL.

Angabe der Resultate unter Berücksichtigung der LOD/LOQ:

Negativ	Keine Signale vorhanden	Der Quantifier oder ein Qualifier mit S/N weniger als 3:1
Negativ Spur	Alle Signale mit S/N $\geq$ 3:1 vorhanden, Wert < LOD	
Positiv	Wert > LOQ und < höchster Kal.	<b>WERT = XXX ng/mL</b>
Positiv RW	Wert > LOD und < LOQ	<b>WERT = XXX ng/mL</b>
Positiv Stark RW	Wert > höchster Kal.	<b>WERT = XXX ng/mL</b>

Konzentrationen, welche  $\pm$  15% vom oberen bzw. unteren Kal. abweichen werden als out bezeichnet. Die Konzentration wird hier als > bzw. < des oberen bzw. unteren Kal. angegeben.

Nachweisgrenze und Bestimmungsgrenze in ng/mL

Resultate [ng/mL]	Phenytoin
LOD	
LOQ	

**Methodenvalidierung**

Siehe Validierungsplan und Validierungsbericht.

Es wurden nur die GC-MS Resultate verwendet. Die Resultate des NPD können in Einzelfällen verwendet werden, um qualitative Resultate vom MSD zu stützen.

**Mitgeltende Unterlagen**

Eingescannte GC-Methode im PIOS  
 Massenspektren von Phenytoin  
 Validierungsplan  
 Validierungsbericht

**Literatur**

Ph. Eur. 5. Ausgabe, Grundwerk 2005, Reagenz Nr. 4010700

Trimethylsulfonium Hydroxide: A New Methylating Agent; Kiyoshi Yamauchi, Toshizumi Tanabe, Masayoshi Kinoshita; J. Org. Chem., Vol. 44, No. 4, 1979

Selective 2-O-Methylation of Pyrimidine-Ribonucleosides by Trimethylsulfonium Hydroxide in the Presece of Mg<sub>2</sub><sup>+</sup> and Ca<sub>2</sub><sup>+</sup> Ions; Kiyoshi Yamauchi, Toshizumi Tanabe, Masayoshi Kinoshita; Bull. Chem. Soc. Jap; Vol. 59; 2947-2949; 1986

## 2. Validierung der Phenytoin-Methode

### 2.1 Validierungsplan

für Phenytoinbestimmung mittels GC-MSD-NPD (Inv. 283)

Bestimmung von freiem Phenytoin im ZNS-Mikrodialysat / Liquor mittels GC-MSD-NPD

### 2.2 Zweck, Prinzip und Bedeutung

Anhand des vorliegenden Validierungsplanes soll überprüft werden, ob die Bestimmung von freiem Phenytoin im ZNS-Mikrodialysat / Liquor mittels GC-MS-NPD (Inv. 283) in der Routineanalytik der Forensischen Toxikologie eingesetzt werden kann und für die definierten Parameter stabil ist. Die Parameter für den Validierungsplan wurden in der Validierungssitzung am 27.5.2008 definiert.

### 2.3 Validierungsparameter

Selektivität

Linearität der Kalibration

Genauigkeit

Stabilität

Analytische Grenzen (Nachweis- und Bestimmungsgrenze)

Wiederfindungsrate, Extraktionsausbeute

Matrixeffekte

Robustheit

### 2.4 Begriffe / Abkürzungen

LSM Lösungsmittel

Kal. Kalibrator

QC Qualitätskontrolle

PP Positivprobe

Leermatrix ohne ISTD Double-Blank, Leerprobe

Leermatrix mit ISTD Blank, Nullprobe

PHT Phenytoin

PDL Phenyhydantoin

MA Mitarbeitende

SPE Festphasen Extraktion

### 2.5 Selektivität (Selectivity)

**Selektivität** ist die Fähigkeit einer Methode, verschiedene nebeneinander zu bestimmende Analyten ohne gegenseitige Störungen oder Störungen durch andere endogene oder exogene Substanzen (Metaboliten, Verunreinigungen, Abbauprodukte, Matrix) zu erfassen und sie somit eindeutig zu identifizieren.

**Spezifität** ist die Fähigkeit einer Methode, einen Analyten oder eine Substanzklasse ohne Verfälschung durch andere in der Probe vorhandene Komponenten (s.o.) zu erfassen und sie somit eindeutig zu identifizieren.

#### **Bestimmung in der Praxis**

Aufarbeitung von mindestens drei verschiedenen Leerproben aus jeweils verschiedenen Chargen (Leermatrix ohne ISTD)

Aufarbeitung von mindestens drei Nullproben [Leermatrix mit ISTD], hergestellt aus humanem ZNS Dialysat, verdünnt mit aCSF (z.B. 50 µL humanes ZNS Dialysat + 450 µL aCSF).

<b>Selektivität</b>	
Vorgehen	- <b>Negativ Proben:</b> Drei Leerproben verschiedener Probanden werden je einmal ohne Zusätze (Double Blank) und einmal mit ISTD-Zusatz (Blank) gemessen.
Proben	- <b>Negativ Proben:</b> Drei verschiedene Leerproben werden verwendet.
Ausführung	R. Hösli
Kontrolle	B. Aebi

## 2.6 Linearität der Kalibration (Linearity of Calibration), nach Ref. [9]

Die Linearität einer analytischen Methode ist ihre Fähigkeit innerhalb eines gegebenen Bereiches Testergebnisse zu liefern, die direkt proportional zur Konzentration (Menge) des Analyten in der Probe sind.

<b>Linearität</b>	
Vorgehen	Überprüfung erfolgt durch Aufarbeitung von jeweils 3 Messserien (durch zwei MA).
Proben	Kal.1 - Kal.6, QC1, QC2, BW ohne ISTD, BW mit ISTD, SST in aCSF BW ist hier gleich aCSF
Ausführung	R. Hösli
Kontrolle	B. Aebi

## 2.7 Kalibrationsbereich (Range)

Der Kalibrationsbereich einer analytischen Methode ist das Intervall zwischen oberer und unterer Konzentration (Menge) des Analyten in der Probe (einschließlich dieser Konzentrationen), für das ein geeignetes Maß an Präzision, Richtigkeit und Linearität gezeigt werden konnte.

### Bestimmung in der Praxis

Herstellung von sechs Kalibratoren (möglichst gleichmässig über den Kalibrationsbereich verteilt) die je doppelt injiziert werden. Es werden 2x 3 Serien (Doppelbestimmung) durchgeführt

## 2.8 Messbereich

Der Messbereich sollte wenn möglich im folgenden Bereich liegen:

<b>Substanz</b>	<b>Messbereich</b>	<b>Therapeutisch auftretender Bereich</b>
PHT	50-1200ng	600-2400ng/mL
PDL	100-1000ng	600-2400ng/mL

## 2.9 Genauigkeit (Accuracy)

Unter Genauigkeit versteht man den Abstand eines einzelnen Wertes vom Sollwert, hervorgerufen durch systematische und zufällige Fehler.

### Bestimmung in der Praxis:

Analyse von mindestens zwei QC-Proben jeder Konzentration an mindestens sechs verschiedenen Tagen, als Doppelbestimmungen. QC1 und QC2 werden je 2x in drei Serien gemessen (Doppelbestimmungen)

<b>Präzision</b> - <b>Wiederholpräzision</b> / - <b>Laborpräzision</b>	
Vorgehen	- <b>Wiederholpräzision:</b> Zwei MA führen je 3 Doppelbestimmung von je 3 Konzentrationen (Kalibratoren Kal.1, Kal.3 und Kal.6) durch. - <b>Laborpräzision:</b> Zwei eingewiesene MA führen jeweils 3 Doppelbestimmung von einer Kontrollprobe durch
Proben	Es werden die Kontrollen QC1 und QC2 und die Kalibratoren Kal.1, Kal.3 und Kal.6 verwendet.
Ausführung	R. Hösli und A. Tobler
Kontrolle	B. Aebi

## 2.10 Stabilität (Stability)

Die chemische Stabilität eines Analyten in einer gegebenen Matrix unter bestimmten Bedingungen für gegebene Zeitintervalle. Die Stabilität eines Analyten sollte vom Zeitpunkt der Probennahme bis zum Abschluss der Analyse gewährleistet sein. Die Stabilität während der Lagerung und während eventuell wiederholtem Einfrieren und Auftauen ist methodenunabhängig, so dass entsprechende Stabilitätsdaten aus der Literatur übernommen werden können.

### 2.10.1 Stabilität aufgearbeiteter Proben (Processed sample stability)

Die Stabilität der (derivatisierten) Analyten in fertig aufgearbeiteten Proben im Probenteller des Autosamplers für die Dauer einer regulären Analysenserie. Die Aufbewahrung während dem Test erfolgt in trockenem Zustand, da das Derivatisierungsmittel (TMSH) verdampfen kann.

### 2.10.2 Bestimmung in der Praxis:

Proben in trockenem Zustand nach Eindampfen lagern und dann kurz vor Messung mit Derivatisierungsmittel versetzen

<b>Robustheit Autosampler-Stabilität / Langzeit-Stabilität</b>	
Vorgehen	<b>Stabilität des aufgearbeiteten Analyten:</b> Die Kontrollen QC1 und QC2 werden sechsmalig aufgearbeitet und abgedampft. Die getrockneten QC's werden aufbewahrt und zum Zeitpunkt 0h (QC1.A und QC2.A), 8h (QC1.B und QC2.B), 24h (QC1.C und QC2.C) mit Derivatisierungsmittel (TMSH) versetzt und sofort gemessen. <b>Langzeit-Stabilität:</b> Wird noch nicht bewertet.
Proben	Kontrollen QC1 und QC2
Ausführung	R. Hösli
Kontrolle	B. Aebi

## 2.11 Analytische Grenzen

### 2.11.1 Nachweisgrenze (Limit of Detection, LOD)

Die Nachweisgrenze ist definiert als die niedrigste Konzentration, die mittels der analytischen Methode noch sicher vom Hintergrundrauschen unterschieden werden kann.

#### Bestimmung in der Praxis:

- Unterster Kalibrator mit S/N (Signal/Noise) 3:1, mittels Dreisatz berechnen
- Reststandardabweichung der Kalibratoren ermitteln
- Nach DIN 32465 vorgehen

### 2.11.2 Bestimmungsgrenze (Limit of Quantification, LOQ)

Die Bestimmungsgrenze ist die niedrigste Konzentration eines Analyten in der Probenmatrix, die mit akzeptablem Bias ( $\pm 20\%$ ) und Präzisionsdaten ( $RSD \leq 20\%$ ) bzw. mit einer vorgegebenen relativen Ergebnisunsicherheit (33%, Signifikanz: 99%) bestimmt werden kann.

#### Bestimmung in der Praxis

- Unterster Kalibrator mit S/N (Signal/Noise) 10:1, mittels Dreisatz berechnen
- Reststandardabweichung der Kalibratoren ermitteln
- Nach DIN 32465 vorgehen

## 2.12 Wiederfindungsrate, Extraktionsausbeute

### 2.12.1 Wiederfindungsrate (Recovery)

Die absolute Wiederfindung ist definiert als kompletter Transfer des Analyten von der Matrix in die zu vermessende Lösung. Sie wird bestimmt aus einem Verhältnis der Signale einer in gleich zugesetzten Menge Analyt bzw. Standard zu einer biologischen Probe und einer nicht extrahierten Originallösung (100%). Die Bestimmung der Wiederfindungsrate bezieht sich immer auf die absoluten Messsignale.

Sie kann daher nur bei Methoden bestimmt werden, bei denen die letztendlich vermessene Substanz in reiner Form erhältlich ist.

### 2.12.2 Wiederfindung in der Praxis

Es wird ein Vergleich mit und ohne Extraktion mit den Kontrollen QC1 und QC2 durchgeführt.

	Mit SPE	Ohne SPE
Wiederfindung in Kontrolle	QC1 (100ng PHT)	QC1 (100ng PHT)
Wiederfindung in Kontrolle	QC2 (1000ng PHT)	QC2 (1000ng PHT)

### 2.13 Robustheit (Robustness, Ruggedness)

Die Robustheit einer analytischen Methode ist ein Maß ihrer Fähigkeit, durch kleine, aber bewusste Veränderungen der Methodenparameter unbeeinflusst zu bleiben, und zeigt ihre Verlässlichkeit während der normalen Anwendung.

Literatur

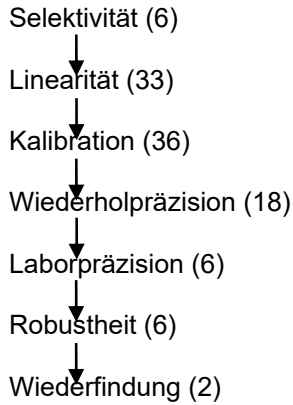
[1] Peters FT, Drummer OH, Musshoff F (2007) Validation of new methods. For.Sci.Int. 165:216-224.

[2] Entwurf zu "Anforderungen an die Validierung von Analysenmethoden", Anhang A, GTFCh, F. T. Peters

### 3. Durchführung der Validierung

Um möglichst effizient und kostengünstig zu arbeiten, wurde eine Zusammenstellung erstellt, aus der ersichtlich wird, aus welchen Messungen welche Daten extrahiert werden können.

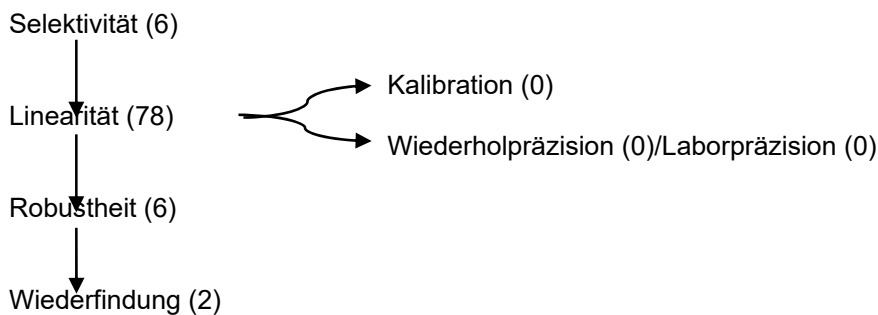
#### 3.1 Anzahl SPE Säulen die pro Schritt verwendet werden müssen



#### 3.2 Anzahl Extraktionen und Messungen bei schrittweise Vorgehen

Untersuchung	Anzahl SPE	Proben
Selektivität (RH)	6	3 Leerproben ohne ISTD 3 Leerproben mit ISTD
Linearität (RH)	18 +12 +6 +3 39	3(Kal 1-Kal6) 3(aCSF ohne ISTD, aCSF mit ISTD) 3(QC1 und QC2) 3(SST)
Kalibration (RH/AT)	36	3(Kal1-Kal6)
Wiederholpräzision (RH/AT)	18	3(Kal1, Kal3, Kal6)
Laborpräzision (RH/AT)	6	3(QC1)
Robustheit (RH)	6	3(QC1 und QC2)
Wiederfindung (RH)	2	QC1 und QC2 mit SPE Extraktion QC1 und QC2 ohne Extraktion
Total SPE's:	113	
Total GS-NPD-MS Messungen	20	1+3+6+6+3+1

#### 3.3 Anzahl SPE Säulen die bei Kombination verwendet werden müssen



**3.4 Anzahl Extraktionen und Messungen bei kombiniertem Vorgehen**

Untersuchung	Anzahl SPE	Proben
Selektivität (RH)	6	3 Leerproben ohne ISTD 3 Leerproben mit ISTD
Linearität (RH /AT)	18 +12 +6 +3 39x2=78	3(Kal 1-Kal6) 3(aCSF ohne ISTD, aCSF mit ISTD) 3(QC1 und QC2) 3(SST)
Kalibration	Aus Linearität	3(Kal1-Kal6)
Wiederholpräzision	Aus Linearität	3(Kal1, Kal3, Kal6)
Laborpräzision	Aus Linearität	3(QC1)
Robustheit (RH)	6	3(QC1 und QC2)
Wiederfindung (RH)	2	QC1 und QC2 mit SPE Extraktion QC1 und QC2 ohne Extraktion
Total SPE's:	92	
Total GC-NPD-MS Messungen	11	1+6+3+1

Gewinn gegenüber schrittweise Vorgehen:

21x SPE Säulen weniger  
9x GC-NPD-MS Messungen weniger

## **STUDY PLAN**

**TITLE OF THE STUDY: VALIDATION OF A METHOD FOR THE  
QUANTIFICATION OF PHENYTOIN IN DIALYZED LIQUOR  
SAMPLES BY LC-MS/MS**



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## ABBREVIATIONS

aCSF	Artificial cerebrospinal fluid
Blank	Artificial cerebrospinal fluid sample processed without internal standard
blq	Below limit of quantification
Cal	Calibration
Conc.	Concentration
CV	Coefficient of variation
Dev	Deviation
DWP	Deep well plate
e.g.	For example
ESI	Electrospray ionization
g	Gram
H <sub>2</sub> O	Water
HClO <sub>4</sub>	Perchloric acid
HCOOH	Formic acid
HPLC	High performance liquid chromatography
ISTD	Internal standard
LC-MS/MS	Liquid chromatography coupled on-line with tandem mass spectrometry
LLOQ	Lower limit of quantification
M	Molarity
MC	Main column
MeCN	Acetonitrile
MeOH	Methanol
mg	Milligram
min	Minute
mL	Milliliter
mm	Millimeter
mM	Millimolar
MS	Mass spectrometry
ng	Nanogram
PHT	Phenytoin
PHT-D10	Phenytoin-D10
PP	Polypropylene
QC	Quality control
SD	Standard deviation
TC	Trapping column
ULOQ	Upper limit of quantification
v + v	Volume plus volume
µm	Micrometer
µg	Microgram
µL	Microliter
%	Percent
°C	Degree centigrade

# **1 GENERAL INFORMATION ABOUT THE STUDY**

Test facility: Institut für Rechtsmedizin, Universität Bern, Bern

Test item: Phenytoin

Title of the study: Validation of a method for the quantification of Phenytoin in Microdialysate matrices by LC-MS/MS.

## **1.1 OBJECTIVE**

The objective of this study is to validate a method for the quantification of Phenytoin in microdialysate samples by LC-MS/MS / HPLC.

## **1.2 PROJECT ADMINISTRATION**

Study director: Raphael Hösli

## **1.3 DEVIATION PROCEDURES**

All deviations to the study plan and the reasons for these deviations will be documented in the raw data. All deviations to the study plan will be described in the study report.

## **1.4 ARCHIVING**

The originals of the study plan, (if applicable), raw data and the final study report will be archived.

## **1.5 VALIDATION OF THE ANALYTICAL METHOD**

The analytical method was developed and will be validated in the test facility. The guideline "Guidance for Industry, Bioanalytical Method Validation, May 2001" (1) will be followed to conduct the validation of the analytical method.

## 2 MATERIALS AND METHODS

### 2.1 TEST ITEMS

Test items will be used for the preparation of Cal and QC samples.

Identity:	Phenytoin
Alternative name:	5.5-Diphenylhydantion, 5.5-Diphenyl imidazolidin-2.4-dion
Certificate of analysis:	Internet CoA
Storage:	at ambient temperature
Formula:	$C_{15}H_{12}N_2O_2$
Molecular weight:	252.28
Purity:	purum, $\geq 96.0\%$
Batch number:	400570/1 33903312

### 2.2 INTERNAL STANDARDS

Internal standards will be used for the preparation of ISTD solutions.

Identity:	Phenytoin D10 (Cerillant)
Certificate of analysis:	will be reported in study report
Storage:	$6^{\circ}C \pm 4^{\circ}C$
Formula:	$C_{15}H_2D_{10}N_2O_2$
Molecular weight:	262.33
Purity:	will be reported in study report
Batch number:	will be reported in study report

### 2.3 BLANK MATRIX

1000 mL blank dilaysate (aCSF) containing NaCl: 8.5910 g, KCl: 0.2013 g,  $CaCl_2$ : 0.1332 g and  $MgCl_2 \cdot 6H_2O$ : 0.1728 g will be used for the preparation of Cal and QC samples. This solution can be self-prepared or purchased from Solna (Sweden). Its pH will be adjusted to 7.4 ( $\pm 0.1$ ) with 2 M NaOH. The aCSF solution will be stored at  $6^{\circ}C \pm 4^{\circ}C$ .

### 2.4 APPARATUS, REAGENTS, AND MATERIALS

All chemicals and equipment can be replaced by equivalent chemicals and equipment.

#### LC-MS/MS system:

Autosampler	Dionex WPS-3000TSL Analy. Olten, Switzerland
Column oven	Cluzeau Info Labo CrocoCil
Column thermostate	Dionex TCC-3100, Olten, Switzerland (including switching valve)
Solvent rack	Dionex SRD-3600, Olten, Switzerland
HPLC pumps 1 and 2	Dionex pump HPG-3200A, Olten, Switzerland
HPLC pump 3	Dionex pump ISO-3100A, Olten, Switzerland
Mass spectrometer	MDS Sciex, Q Trap 3200, Toronto Canada

Nitrogen Liquid Nitrogen, Messer, Lenzburg, Switzerland

**Other equipment:**

Balances AE163 Mettler-Toledo; XS603S Mettler-Toledo  
 Centrifuges Rotanta 460 R and / or Mikro 22R, Hettich Instruments, Andreas Hettich AG, Bäch, Switzerland  
 Deep well plates 0.6 mL, Chemie Brunschwig AG, Basel, Switzerland and / or 1 mL, Vitaris, Baar, Switzerland and / or Chemie Brunschwig AG, Basel, Switzerland and / or 1.2 mL, TreffLab, Milian S.A., Geneva, Switzerland  
 HPLC vials 2.0 mL glass, BGB Analytik AG, Böckten, Switzerland  
 Inserts 0.3 mL glass, BGB Analytik AG, Böckten, Switzerland  
 Pipetman tips Axygen, Milian S.A., Geneva, Switzerland  
 Pipettes Eppendorf, Milian S.A., Geneva, Switzerland, Socorex ISBA S.A., Lausanne, Switzerland and Gilson, Mettmenstetten, Switzerland  
 PP tubes Various sizes of safe seal tubes and / or screw cap tubes, brown and / or transparent, 1.5 to 50 mL, Sarstedt, Sevelen, Switzerland and / or Milian S.A., Geneva, Switzerland  
 Sealing mats Silicone, Chemie Brunschwig AG, Basel, Switzerland and / or Corning, Vitaris AG, Baar, Switzerland  
 Glas flasks with caps Various sizes, 10 to 2000 mL, VWR International, Dietikon, Switzerland and 2.0 and / or 4.0 mL, BGB Analytik AG, Böckten, Switzerland  
 Vortex mixer Vibrax-VXR, IKA, Fisher Scientific AG, Wohlen, Switzerland

**HPLC columns:**

Trapping column Phenomenex Gemini Polar, 2.0 x 10 mm, 5 µm, Brechbühler AG, Schlieren, Switzerland  
 Main column Phenomenex Synergy Polar RP, RP, 2.0 x 50 mm, Brechbühler AG, Schlieren, Switzerland

**Chemicals:**

Acetonitrile HPLC grade, Chemie Brunschwig AG, Basel, Switzerland  
 Methanol HPLC Gradient grade, Chemie Brunschwig AG, Basel, Switzerland  
 Perchloric acid 20% purissimum pro analysi, Fluka Chemie GmbH, Buchs, Switzerland  
 Water MiliQ quality, Milpore

**Software:**

Analyst software Version 1.5.x, AB Sciex, Toronto, Canada  
 Microsoft Office Version 2007

## 2.5 DESCRIPTION OF THE HPLC SYSTEM

### Column switching / description of the method:

Flow pumps 1	0.350 mL/min
Flow pump 2	0.200 to 1.000 mL/min
Oven temp:	set to 50°C
Autosampler cooler tray:	set to 8°C
Syringe volume:	100 µL
Injection loop:	130 µL
Injection volume:	1 - 100 µL
Mobile phase A:	H <sub>2</sub> O + HCOOH (100 + 0.1, v + v)
Mobile phase B:	MeCN + HCOOH (100 + 0.1, v + v)

**Table 1:** Pump gradient program

Pump 1 (mobile phase A)			Pump 2 (mobile phase A and mobile phase B)				Pump 3 (mobile phase A)
Time (min)	B%	Flow (mL/min)	Time (min)	B%	Flow (mL/min)		Flow (mL/min)
0.00	10	0.350	0.00	100	0.20	Trapping	2.00
1.00	10	0.350	0.00	100	0.20		2.00
2.00	97.5	0.350	1.00	100	0.20	Eluting	2.00
4.00	97.5	0.350	1.20	100	0.02		2.00
4.10	10	0.350	3.50	100	0.02		0.02
5.00	10	0.350	3.60	100	1.00		0.02
			4.50	100	1.00		0.02
			4.60	100	0.02		2.00
			5.00	100	0.02		2.00

### Retention times of the analytes:

Phenytoin:	approximately 2.8 min
ISTD (PHT D10):	approximately 2.8 min

### Autosampler washing solutions:

First solution:	MeCN + HCOOH (100 + 0.1, v + v)
-----------------	---------------------------------

## 2.6 DETECTOR SETTINGS

### Mass spectrometry settings:

Source interface:	Electrospray ionization
Polarity:	Positive
Acquisition time (per sample):	5.5 min
Scan type:	MRM mode

The *m/z* values of the different ions that will be used to measure the concentrations of the analyte and ISTDs in worked-up matrix samples are listed in [Table 2](#).

**Table 2:** m/z values to be used for quantification in MRM mode

	Precursor ion m/z	Fragment ion m/z	Dwell time (ms)
Phenytoin	253	182	100
ISTD (PHT-D10 )	263	192	100

## 2.7 DATA ACQUISITION AND CALCULATION

Sample lists, acquisition method and data collection will be generated with Analyst version 1.5.x, AB Sciex, Toronto, Canada. The acquired data will be processed with Analyst version 1.5.x, AB Sciex, Toronto, Canada. The software is capable of smoothing peaks, drawing baselines, calculating peak heights and areas and determining concentrations by inverse prediction and statistical evaluation of the Cal curve fit.

The concentrations of Phenytoin in Cal and QC species matrix samples will be back calculated from the Cal curves using the internal standardization method. Analyst versions 1.5.x, AB Sciex, Toronto, Canada will be used to calculate a linear regression from the area ratio and the corresponding concentration data of the Cal samples.

For Cal and QC samples the accuracy and precision of the validation runs will be calculated by Microsoft Excel.

## 2.8 DATA REPORTING

Statistical data for mean, overall mean and SD will be rounded according to the decimals given for the analytes. Statistical data for CV%, Dev% and accuracy will be rounded to 1 decimal. Due to the rounding procedures, reported values may differ from the original raw data. Statistical evaluations will be made based on rounded matrix concentrations.

## 2.9 FORMULAE

### 2.9.1 Deviation

Intra- and inter-assay deviation (Dev%) will be calculated for all determinations using the following equation:

$$\text{Dev}\% = 100 * \frac{(c_m - c_n)}{c_n}$$

Explanation of the symbols used in this equation:

Dev% =	Deviation of measured concentrations from the nominal concentration in %
$c_m$ =	Measured concentration in [ng/mL]
$c_n$ =	Nominal concentration in [ng/mL]



### 2.9.2 Mean

Mean ( $\bar{x}$ ) will be calculated using the following equation:

$$\bar{x} = \frac{\sum_{i=1}^n x_i}{n}$$

Explanation of the symbols used in this equation:

$\bar{x}$ =	Mean or overall mean concentration of specified Cal and QC levels of all valid determinations
$x_i$ =	Concentration of specified Cal and QC levels
$n$ =	Number of assays

### 2.9.3 Standard deviation

Standard deviation (SD) will be calculated for all determinations using the following equation:

$$SD = \sqrt{\frac{n \sum_{i=1}^n x_i^2 - \left( \sum_{i=1}^n x_i \right)^2}{n(n-1)}}$$

Explanation of the symbols used in this equation:

SD =	= Standard deviation
$x_i$ =	= Concentration of specified Cal and QC levels
$n$ =	= Number of assays

### 2.9.4 Accuracy

Accuracy in % will be calculated for all determinations using the following equation:

$$\text{Accuracy \%} = \frac{\bar{x}}{c_n} * 100$$

Explanation of the symbols used in this equation:

Accuracy % =	Accuracy in % of nominal concentration
$\bar{x}$ =	Mean or overall mean concentration of specified Cal and QC levels from all valid determinations
$c_n$ =	Nominal concentration

### 2.9.5 Intra- and inter-assay reproducibility / precision

Intra- and inter-assay precision (CV%) will be calculated for all determinations using the following equation:

$$CV\% = \frac{SD}{\bar{x}} * 100$$

Explanation of the symbols used in this equation:

SD =	Standard deviation in %
$\bar{x}$ =	Mean or overall mean concentration of specified Cal and QC levels of all determinations

## 3 EXPERIMENTAL PROCEDURE

If any different amounts and / or volumes than indicated below will be used during the experimental part the corresponding amounts and / or volumes have to be adjusted. Preparations described in this chapter will be mixed well, if not mentioned to be handled else, before being used for the next step.

### 3.1 PREPARATION OF STOCK SOLUTIONS

The concentrations of the solutions are based on the free and non-ionized form of the drug. All solutions will be prepared in PP tubes / glass volumetric flasks / single use glass flasks under consideration of the content.

#### **Solution KA (1.00 mg/mL of Phenytoin):**

Accurately weigh at least 10.00 mg of Phenytoin and dissolve in 10.00 mL MeOH in order to achieve a concentration of 1.00 mg/mL.

#### **Solution SA (1.00 mg/mL of Phenytoin):**

Accurately weigh at least 10.00 mg of Phenytoin and dissolve in 10.00 mL MeOH in order to achieve a concentration of 1.00 mg/mL.

#### **Stock solution ISTD (100 µg/mL in MeOH):**

1.0 mL ampoule of Phenytoin-D10 (Cerilliant).

The preparations above will be shaken until dissolution is complete. Subsequently, they will be stored 6°C ± 4°C.

### 3.2 PREPARATION OF CALIBRATION SAMPLES

Eight Cal samples with a concentration range from 10.00 to 2000 ng/mL for Phenytoin will be prepared in blank aCSF from stock solutions KA (Table 3)

**K:** 1.00 mg/mL = 1'000 µg/mL = 1'000'000 ng/mL Phenytoin

Table 3: Preparation of calibration samples

<b>Working solutions</b>	<b>C8</b>	<b>C7</b>	<b>C6</b>	<b>C5</b>	<b>C4</b>	<b>C3</b>	<b>C2</b>	<b>C1</b>
[ng/mL]	100'000	50'000	25'000	12'500	5'000	2'500	1'000	500.0
MeOH mL	1.80	1.00	1.00	1.00	1.20	1.00	1.20	1.00
From solution	K	C8	C7	C6	C5	C4	C3	C2
Add mL	0.20	1.00	1.00	1.00	0.80	1.00	0.80	1.00
<b>Calibration samples</b>	<b>Cal8</b>	<b>Cal7</b>	<b>Cal6</b>	<b>Cal5</b>	<b>Cal4</b>	<b>Cal3</b>	<b>Cal2</b>	<b>Cal1</b>
aCSF(µL)	980	980	980	980	980	980	980	980
From solution	C8	C7	C6	C5	C4	C3	C2	C1
Add (µL)	20	20	20	20	20	20	20	20
Conc. of Phenytoin [ng/mL]	2000	1000	500.0	250.0	100.0	50.00	20.00	10.00

Working solutions will be stored in PP tubes at 6°C ± 4°C.

### 3.3 PREPARATION OF QUALITY CONTROL SAMPLES

Quality control samples at 5 different concentrations of Phenytoin will be prepared in blank aCSF from stock solutions SA (Tables 4). 10xQC<sub>high</sub> samples will be prepared for dilution ability (see 5.6).

**SA:** 1.00 mg/mL = 1000 µg/mL = 1'000'000 ng/mL Phenytoin

Table 4: Preparation of quality control samples

<b>Working solutions</b>	<b>Q4</b>	<b>Q3</b>	<b>Q2</b>	<b>Q2#</b>	<b>Q1</b>	<b>Q<sub>LLOQ</sub></b>
[ng/mL]	800'000	80'000	20'000	6000	1500	500
MeOH(µL)	80	1840	1500	1400	900	400
From solution	SA	SA	Q3	Q2	Q2#	Q1
Add(µL)	320	160	500	600	300	200
<b>Quality control samples</b>	<b>10 xQC<sub>high</sub></b>	<b>QC<sub>high</sub></b>	<b>QC<sub>medium</sub></b>		<b>QC<sub>low</sub></b>	<b>QC<sub>LLOQ</sub></b>
aCSF	490	980	980		980	980
From solution	Q4	Q3	Q2		Q1	Q <sub>LLOQ</sub>
Add (µL)	10	20	20		20	20
Conc. of Phenytoin [ng/mL]	16000	1600	400		30	10

Working solutions will be stored in PP tubes at 6°C ± 4°C.

Blank: aCSF sample free of analytes and processed without ISTD as described in the sample preparation procedure.  
 QC0: aCSF sample free of analytes and processed with ISTD as described in the sample preparation procedure.

### 3.4 PREPARATION OF INTERNAL STANDARD SOLUTIONS

**ISTD stock solution:**

100 µg/mL ampoule of Phenytoin-D10 (Cerilliant).

**ISTD1 solution:** 100 µL of the 100 µg/mL is added to 9900 µL of MeOH. The solution will be mixed well by shaking.

**ISTD working solution:** 5 mL of the ISTD1 is added to 95 mL of a 1 M perchloric acid aqueous solution. The solution will be mixed well by shaking.

Subsequently the solutions will be stored at 6°C ± 4°C [50ng/mL]

### **3.5 SAMPLE PREPARATION**

Sample preparation for 25 µL of Blank, Cal and QC dialysate samples:

1. The individual Blank, Cal and QC aCSF samples will be thawed in cold tap water or at ambient temperature.
2. 25 µL of each sample will be aliquoted or previously aliquoted samples will be used.
3. 75 µL of ISTD working solution is added.
4. The samples will be centrifuged at about 4.000 u/min for about 30 minutes at  $6^{\circ}\text{C} \pm 4^{\circ}\text{C}$ .
5. Samples will be ready for injection into the LC-MS/MS system.

All worked-up samples will always be stored at  $6^{\circ}\text{C} \pm 4^{\circ}\text{C}$  if not used immediately.

## **4 VALIDATION: DESCRIPTION AND CRITERIA**

### **4.1 SELECTIVITY**

For selectivity 6 dialysate samples will be prepared and analyzed as Blank samples. The mean interference of these 6 samples has to be beyond 1/5 of the analytes' mean response at the  $\text{QC}_{\text{LLOQ}}$  level.

### **4.2 SPECIFICITY**

For specificity, 6 dialysate samples will be spiked with Phenytoin at the level of the LLOQ, then worked-up and analyzed as  $\text{QC}_{\text{LLOQ}}$  samples.

The mean accuracy has to be within  $\pm 20\%$  of the nominal concentration. The mean precision of the 6 samples has to be within 20%.

### **4.3 VALIDATION RUNS FOR ACCURACY AND PRECISION**

#### **4.3.1 Composition of a validation run for accuracy and precision**

A validation run will include 2 sets of Cal samples 1 to 8 for Phenytoin, 2 Blanks, 2 QC0 samples and a set of 6 QC samples at each of the 4 different concentrations ( $\text{QC}_{\text{LLOQ}}$ ,  $\text{QC}_{\text{low}}$ ,  $\text{QC}_{\text{medium}}$  and  $\text{QC}_{\text{high}}$ ). Three of these validation runs will be separately prepared and measured.

Standard deviation and CV% will be calculated for each Cal and QC level with more than 1 result. Deviation in %, mean and accuracy in % will be evaluated for all valid results. All valid validation runs will be used for the calculation.

#### **4.3.2 Accuracy**

Accuracy for Cal samples will be calculated as inter-assay (between different runs) from the overall mean at each Cal level divided by the corresponding nominal concentration. Accuracy for QC samples will be calculated as intra-assay (within a run) and inter-assay from the mean

and overall mean at each QC level divided by the corresponding nominal concentration. Intra- and inter-assay accuracy have to be within  $\pm 20\%$  at the lowest Cal and QC level and within  $\pm 15\%$  at all other Cal and QC levels.

#### **4.3.3 Precision**

Precision for Cal samples will be determined as inter-assay reproducibility. Precision for QC samples will be determined as intra- and inter-assay reproducibility. Inter-assay precision has to be within 20% at the lowest Cal level, intra- and inter-assay precision have to be within 20% at the lowest QC level and within 15% at all other QC levels.

#### **4.3.4 Limits of quantification**

The lowest Cal level (Cal1) will be used as the LLOQ and the highest Cal level (Cal8) will be used as the ULOQ.

The LLOQ in dialysate samples will be set to 10.00 ng/mL and the ULOQ to 2000 ng/mL for Phenytoin.

#### **4.3.5 Calculation of calibration samples**

Version 1.5.x, AB Sciex, Toronto, Canada will be used to perform a linear regression by plotting area ratio of Phenytoin versus (ISTD) against the corresponding ratios of Phenytoin nominal concentration versus ISTDs nominal concentrations. During method validation the weighting factor giving the best fit will be evaluated and selected for the linear regression. All Cal samples which fulfill the specifications will be used to generate the standard Cal curve. This means that for a valid run a standard Cal curve will include at least 12 to a maximum of 16 Cal samples. Calibration samples which will be out of specifications will not be used for any further calculations.

#### **4.3.6 Acceptance criteria for the calibration curve**

- Analyte response at the LLOQ should be at least 5 times the response of the Blank.
- The deviation of the lowest Cal samples from the nominal concentration has to be within  $\pm 20\%$ .
- The deviation of the other Cal samples from the nominal concentration has to be within  $\pm 15\%$ .
- At least 75% of all Cal samples (including at least 1 of the highest and 1 of the lowest Cal samples) have to fulfill the above criteria.
- The correlation coefficient for the Cal curve linearity has to be  $\geq 0.99$ .
- The inter-assay accuracy and precision at the lowest Cal level from the nominal concentration have to be within ( $\pm$ ) 20% while the inter-assay accuracy for the remaining Cal levels has to be within  $\pm 15\%$ .

If a run will not fulfill the criteria the run will be re injected and / or completely reanalyzed.

#### **4.3.7 Calculation of quality control samples**

The standard Cal curve will be used to back calculate the concentrations of Phenytoin in QC samples by using the measured corresponding area ratio. All results for the QC samples except for QC samples which deviate from the nominal concentration due to an error that may occur will be used for the calculation.

#### 4.3.8 Acceptance criteria for quality control samples

- The intra- and / or inter-assay accuracy and precision at the lowest QC level from the nominal concentration have to be within ( $\pm$ ) 20% while the intra- and / or inter-assay accuracy and precision for the remaining QC levels have to be within ( $\pm$ ) 15%.
- QC0 samples should be blq.
- If for Blank samples possible interfering peak(s) with the analyte(s) occurs, the peak area(s) of this / these peak(s) should be  $\leq$  the area of an interfering peak(s) of the QC0 samples.

If a run will not fulfill the criteria the run will be reinjected and / or completely reanalyzed.

#### 4.4 RECOVERY

For recovery 18 blank dialysate samples will be worked-up and spiked with Phenytoin as well as ISTD at the actual concentrations received in QC<sub>low</sub>, QC<sub>medium</sub> and QC<sub>high</sub> samples after work-up. For each of the 3 levels 6 replicates will be prepared. Additionally 6 original samples of each of the levels QC<sub>low</sub>, QC<sub>medium</sub> and QC<sub>high</sub> will be worked-up.

The mean area of the spiked Blank samples will be compared with the mean area of the original QC<sub>low</sub>, QC<sub>medium</sub> and QC<sub>high</sub> samples at each level. The deviation between the mean areas at each level will be used as recovery for Phenytoin as well as for ISTD. The intra-assay precision should be consistent and precise (CV%  $\leq$  25).

#### 4.5 STABILITY TESTS

For the short-term, freeze-thaw and the long-term stability a set of 12 dialysate samples - 6 of each concentration at QC<sub>low</sub> and QC<sub>high</sub> level - will be used.

Except for the long-term stability test the nominal concentration of the QC<sub>low</sub> and QC<sub>high</sub> samples will be used as reference.

The intra-assay accuracy and precision of the analyte's/analytes' mean concentrations in the QC samples at QC<sub>low</sub> and QC<sub>high</sub> level have to be within  $\pm$  15% of their nominal concentration for short-term and freeze-thaw respectively as described from the actual level for the long-term stability tests.

##### 4.5.1 Freeze-thaw stability

Analyte stability will be determined for 3 freeze-thaw cycles. Samples will be stored at  $-25^{\circ}\text{C} \pm 5^{\circ}\text{C}$  for at least 24 hours and then thawed unassisted at ambient temperature. When completely thawed, the samples will be refrozen and stored for at least 12 hours at the mentioned temperature. The freeze-thaw cycle will be repeated twice. After the 3<sup>rd</sup> cycle the samples will be worked-up and analyzed. If this test shows, that the analyte is not stable for 3 freeze-thaw cycles, the test will be repeated for 1, 2 and 3 freeze-thaw cycles.

##### 4.5.2 Short-term stability

The samples will be thawed at ambient temperature and kept at this temperature for at least 6 hours, frozen for at least 12 hours at  $-25^{\circ}\text{C} \pm 5^{\circ}\text{C}$ , then thawed, worked-up and analyzed

##### 4.5.3 Long-term stability

For the evaluation of the long-term stability QC<sub>low</sub> and QC<sub>high</sub> samples will be spiked. A set of 6 replicates will be measured immediately for each level. The mean of the measured

concentrations at each level will be used as the reference concentration for the stored QC samples.

Long-term stability will be determined for a period of at least 3 and 6 months. Therefore freshly prepared QC samples will be stored at  $-25^{\circ}\text{C} \pm 5^{\circ}\text{C}$ .

After at least 3 and 6 months of storage the QC samples will be analyzed against freshly prepared Cal samples.

#### **4.5.4 Stock solution stability**

Stock solutions of Phenytoin will be kept for at least 6 hours at ambient temperature before being stored at  $-25^{\circ}\text{C} \pm 5^{\circ}\text{C}$  for at least 6 months.

After completion of each of the storage periods the peak areas of samples prepared using stored stock solutions will be compared with the peak areas of samples prepared using freshly prepared stock solutions.

The intra-assay precision has to be within  $\pm 15\%$  for each stock solution. The deviation between the mean areas of the samples of stored and freshly prepared stock solutions has to be within  $\pm 15\%$  for each analyte.

#### **4.5.5 Post-preparative stability / reinjection stability**

Post-preparative stability will be evaluated to determine if an analytical run can be reinjected in the case of instrument failure. Therefore 1 of the validation runs (see 5.3) will be analyzed a second time after at least 1 day and maximum of 7 days. The described criteria for Cal curves, QC, accuracy and precision have to be met.

The mean of the analyte's concentrations in the species1 matrix has to be within  $\pm 15\%$  from the mean of the analyte's concentrations of the  $t_0$  samples.

### **4.6 SAMPLE DILUTION ABILITY**

The validity to dilute samples which will be found to be above the ULOQ after the first analysis during a study will be demonstrated by diluting  $10 \times \text{QC}_{\text{high}}$  samples to  $\text{QC}_{\text{high}}$  samples with blank dialysate.

### **4.7 CARRY OVER**

The carry over of Phenytoin will be determined with a test sequence of 1 Blank sample followed by 1 Cal8 sample and 4 Blank samples. The analyte's peak area of the Cal8 will be compared with the analytes' peak area of the Blank samples. The test sequence will be repeated 3 times.

## **5 REPORTING**

**The final report will include, but not be limited to:**

- The identification of the study and test items (including characteristics)
- The study schedule
- A description of all materials and methods used



- A description of all results (concentrations of the Cal and QC samples, accuracy, precision, linearity)
- The storage locations of study plan, raw data, final report, and reference samples of the test items

## **6 REFERENCES**

- (1) FDA Guidance for Industry (Bioanalytical Method Validation, May 2001); (<http://fda.gov/cder/guidance/index.htm>).

## **7 DISTRIBUTION**

Original:	Archive
Copies:	Study director

## ***Appendix C: Poster Presentations***

# Determination of Free Phenytoin Blood Concentrations in Patients: Measured Versus Calculated Serum Levels

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## Introduction:

Total drug concentration in blood/plasma is routinely determined in difficult-to-dose drugs therapeutic drug monitoring or (TDM). To adapt correct dosing in patients with changing drug-binding proteins like albumin, the knowledge of the free fraction of highly protein-bound drugs is required. Phenytoin (PHT), an antiepileptic with narrow therapeutic index, >90% albumin binding and a  $V_d$  of 0.7 L/kg b.w. needs dose individualization because of its non-linear pharmacokinetics with a slow dose-dependent elimination. Therefore, PHT TDM is mandatory for rapid i.v. loading and subsequent correct dosing as of shown in a study comparing Bayesian forecasting with conventional dosing in a tertiary care hospital [1].

## Aims:

The aim of the study is to evaluate the utility of the Sheiner-Tozer equation for the free serum PHT level assessment in a hospital setting. We selected patients with low serum albumin < 35 g/L in whom the total and the free serum PHT concentrations were measured and compared the measured free PHT levels with the calculated ones.

## Methods:

23 adult patients with a hypo-albuminaemia could be selected from a total of >2500 patients [1]. They were subdivided in 2 groups: the very low Albumin (alb < 25 g/L; n=12) and the low albumin group (35 > alb ≥ 25 g/L; n=11). Albumin and total PHT (dphT), determined with a routine EMIT lab assay, were analyzed in the central lab of the Kantonsspital Aarau. The free (dphEF) levels were measured in a specialized lab for antiepileptics (Epi Klinik Zürich) by using the supernatant of the plasma sample after ultracentrifugation (30 kD cutoff, 20 min centrifugation time) and by a HPLC method. The Sheiner-Tozer equation was used to calculate the free PHT from dphT [µg/ml] and the albumin concentration [g/100 mL] (Figure1):

$$dphEF = \frac{dphT \times 0.1}{0.9 \times \frac{Albumin [g/100mL]}{4.4} + 0.1} \quad [\mu g/mL]$$

Figure 1: The Sheiner-Tozer formula

Free PHT (dphEF) [g/L] is calculated from the total PHT (dphT) and the serum albumin concentration; 4.4 represents the mean serum albumin value [g/100mL]. 0.9 corrects for the protein bound PHT fraction (90%); 0.1 for the free PHT fraction (10%).

The calculated and the measured free PHT fraction were compared using Sperman Rho (non-parametric) statistics together with mean, median, and Standard Deviation (SD) calculations.

## Results:

The two methods correlated very well (Sperman's Rho 0.907, p=0.000; n=23) and showed no significant differences (Figure2). The differences between measured and calculated values were < 6.5%.

The results were comparable in the low and the very low albumin group with max. deviation between the measured and the calculated free fraction of < 6.39% (SD = 1.4; median = 3.63) and < 6.17% (SD = 1.88; median = 2.80) respectively.

In Table 1 the values of the calculated total PHT levels and the free PHT fraction for the investigated subjects are depicted. The one way ANOVA as well as the T-test showed no difference in the two groups.

ANOVA p-value = 0.129, critical f-value = 4.32;  
 T-test: p-value = 0.129, critical t-value = 2.08.

## Keywords:

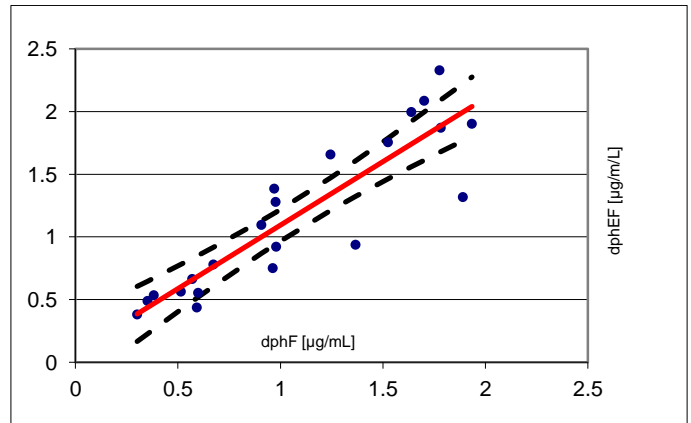
Free phenytoin serum concentrations, Sheiner-Tozer equation.

## References:

[1] Tobler A, Mühlebach S. Int J Clin Pharm 2013; 35: 790-7.

Figure 2: Free PHT: measured (dphF) vs. calculated (dphEF)

Regression analysis (n=23) between free PHT and calculated free PHT. (Albumin < 35g/L)



Linear regression: — 95% confidence interval: - - - - Sperman's rho 0.907 (p=0.000)

Table 1: Individual data per patient including statistics (n=23)

Total PHT levels and the free PHT fraction; measured and calculated (Sheiner-Tozer equation) values. SD: Standard deviation, MeanDev is the mean (absolute) deviation from the Mean Value.

Nr.	total PHT level [mg/l] [dphT]	analysed free PHT fraction [dphF]	calculated free PHT fraction [dphEF]	[dphF] in % of [dphT]	[dphEF] in % of [dphT]	Difference [dphEF % - dphF %]	Albumin [g/L]
1	2.58	0.30	0.38	11.70	14.86	3.16	28
2	3.20	0.35	0.49	11.00	15.33	4.33	27
3	3.83	0.38	0.54	10.00	14.01	4.01	30
4	2.88	0.51	0.56	17.90	19.64	1.74	20
5	4.75	0.57	0.67	12.00	14.01	2.01	30
6	2.50	0.59	0.44	23.70	17.53	6.17	23
7	2.83	0.60	0.55	21.20	19.64	1.56	20
8	4.78	0.67	0.78	14.10	16.36	2.26	25
9	8.73	0.91	1.10	10.40	12.57	2.17	34
10	3.68	0.96	0.75	26.20	20.47	5.73	19
11	11.03	0.97	1.39	8.80	12.57	3.77	34
12	8.35	0.98	1.28	11.70	15.33	3.63	27
13	5.08	0.98	0.92	19.30	18.18	1.12	22
14	9.80	1.24	1.66	12.70	16.92	4.22	24
15	6.70	1.37	0.94	20.40	14.01	6.39	30
16	10.38	1.53	1.76	14.70	16.92	2.22	24
17	10.58	1.64	2.00	15.50	18.88	3.38	21
18	11.05	1.70	2.09	15.40	18.88	3.48	21
19	16.15	1.78	2.33	11.00	14.43	3.43	29
20	10.68	1.78	1.87	16.70	17.53	0.83	23
21	9.95	1.89	1.32	19.00	13.25	5.75	32
22	9.30	1.93	1.90	20.80	20.47	0.33	19
23	14.40	1.93	2.44	13.40	16.92	3.52	24
SD	4.0	0.6	0.7				
Median	8.4	1.0	1.1				24.0
Mean	7.5	1.1	1.2				25.5
Mean Dev	3.5	0.5	0.6				4.0

One way Anova (free PHT values for Patient with: albumin <25 (n=12) compared to albumin ≥25 (n=11))

p-value	critical f-value	f-value
0.129	4.32	2.50
variance group <25:	0.289	variance group ≥25:
		0.306

Two-sample t-Test (free PHT values for Patient with: albumin <25 (n=12) compared to albumin ≥25 (n=11))

p-value	critical t-value	t-statistic
0.129	2.08	1.58

## Conclusion:

From this study we conclude that the free PHT serum concentration can be calculated by the Sheiner-Tozer equation in hospitalized patients with hypo-albuminaemia (19-35 g/L) with the appropriate precision. Thus, the Sheiner-Tozer method represents an useful, quick, and almost free of cost bedside approach.

## Introduction:

Phenytoin (PHT) is indicated to treat and/or prevent epilepsy in neurosurgical patients. Little is known about the fate of PHT in the brain (target organ) and the correlating concentrations in other biological samples commonly used for TDM like serum. To investigate this particular pharmacokinetic correlation, a sensitive, reliable, and cost-effective analytical method has to be established and validated. GC-MS and LC-MS are possible methods to quantify PHT in biological samples. Therefore, the two methods were elaborated and validated for PHT using samples with concentrations expected in patients. Analytical data from ex vivo CNS microdialysis, artificial cerebrospinal fluid (aCSF), blood and saliva were compared for both methods.

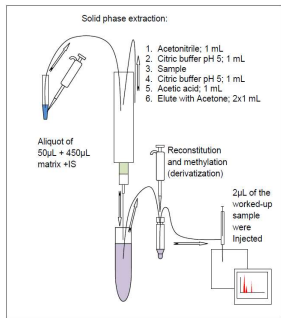


Fig 1: Clean-up of samples for GC-MS analyses (SPE).

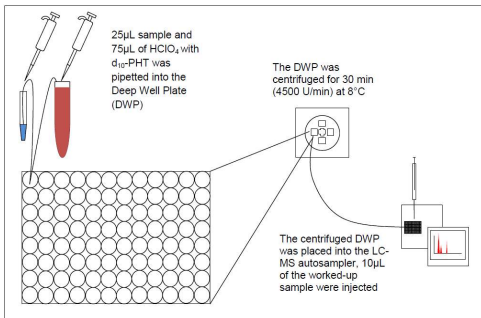


Fig 2: Sample preparation in deep well plates for LC-MS analyses for blood, saliva and aCSF samples.

## Method:

The GC-MS analysis needs a solid phase extraction (SPE) for clean-up of the samples (Fig 1). Derivatization of the residue was made with trimethylsulphonium hydroxide. As internal standard (IS), 5-(p-Methylphenyl)-5-phenylhydantoin (MPPH) was used. The calibration curve ranged from 50 to 1200 ng/mL. For the LC-MS analysis,  $d_{10}$ -PHT (100 µg/mL in MeOH) was used as IS, diluted with  $HClO_4$  to 50 ng/mL. 75 µL of this solution was added to 25 µL sample for perchloric acid precipitation (Fig 2). The calibration curve ranged from 10 to 2000 ng/mL PHT. Both methods were validated according to ISO / FDA Guidance for Industry. Selectivity, sensitivity, recovery, limit of detection (LOD), limit of quantification (LOQ), accuracy, linearity, and extract stability of both procedures were assessed. The performance of the two methods were compared: sample volume needed, analysis time and costs.

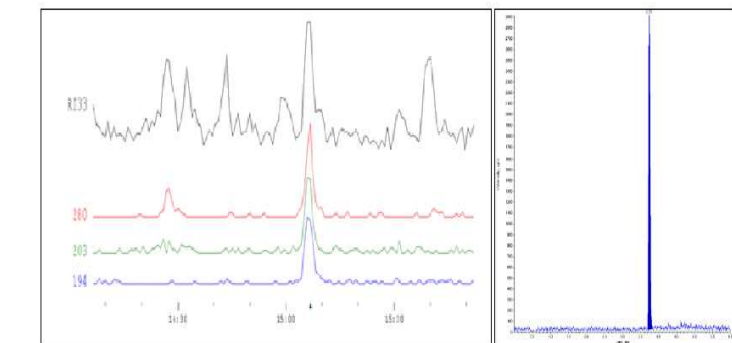


Fig 3: GC-MS 50 ng/mL PHT (Cal1) black = TIC chromatogram; red, green, and blue chromatograms of the mass fragments at m/z 280, 203 and 194

Fig 4: LC-MS 10 ng/mL (Cal1), PHT

## Conclusions:

- The LC-MS method shows better performance in terms of sample consumption, sample preparation time, run time per sample, robustness and linearity (Fig 7). Accuracy and precision are comparable.
- For LC-MS the linearity of the calibration covers a larger range (10 – 2000 ng/mL), covering the expected PHT concentrations in patients.
- For larger clinical studies LC-MS is the preferred method compared to GC-MS.

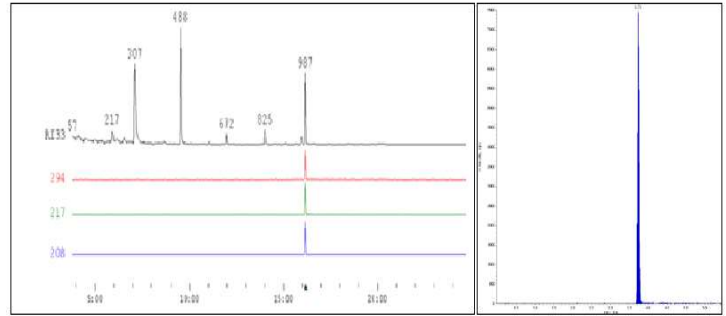


Fig 5: GC-MS Internal standard 1200 ng/mL MPPH

Fig 6: LC-MS Internal standard 50 ng/mL  $d_{10}$ -PHT

## Results:

### GC-MS:

Retention time: PHT = 15.12min (Fig 3), MPPH = 16.15 (Fig 5). Selectivity and sensitivity: all blank samples were negative. Recovery: Quality control (QC) 100 ng/mL, QC 1000 ng/mL ≤ 8% min/max deviation from target value. LOD = 15 ng/mL, LOQ = 50 ng/mL for all matrices. Accuracy: 1-10% for calibrator 2 (Cal2) (150 ng/mL) to Cal6 (1200 ng/mL) and 20% for Cal1 (50 ng/mL). The calibration curve was linear ( $r^2 \geq 0.995$ , aCSF \*n=8, blood \*n=2, and saliva \*n=2). Dried extracts were stable ≥ 4 weeks (min/max deviation 4%). Reinjection and storage (33h) on the autosampler showed no variations. The run time was 30 min. per analysis; the clean-up time for 25 samples took 5 h. The cost for materials and consumables was about 8 CHF per sample.

### LC-MS:

Retention time for PHT and  $d_{10}$ -PHT were at about 2.8 min. (Fig 4 and Fig 6). Selectivity and sensitivity: for blank samples no interferences were detected. Recovery: min/max deviations from target value were 10% for the lowest QC and 3% for the highest QC. LOD << 10 ng/mL, LOQ = 10 ng/mL. Accuracy: 1-8% for Cal2 (20 ng/mL) to Cal8 (2000 ng/mL) and 3% for Cal1 (10 ng/mL). The calibration curve was linear ( $r^2 \geq 0.997$ , aCSF \*n=6, blood \*n=3, and saliva \*n=3). Reinjection after 7 days; no difference in accuracy was detectable. The run time is 7 min. per analysis, preparation time for 182 samples took 6 h. (\*n = number of calibration curves)

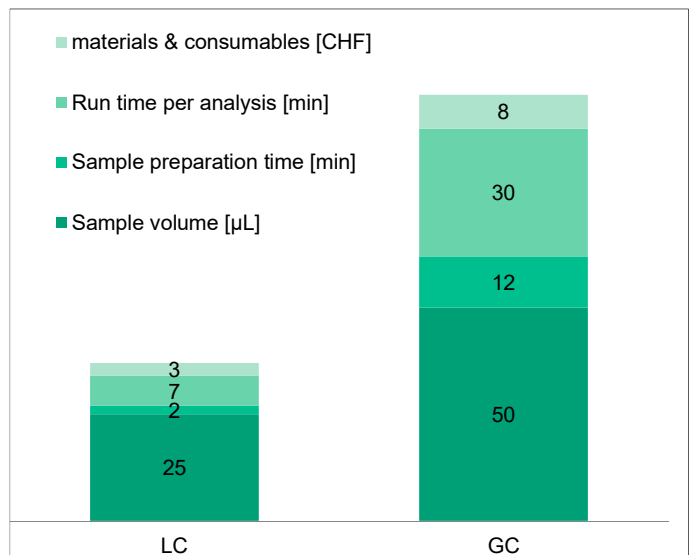
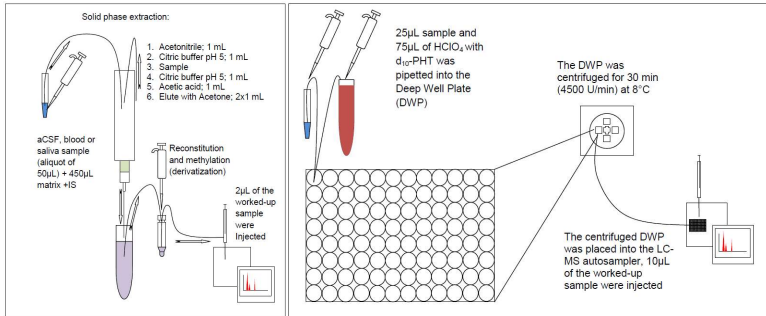


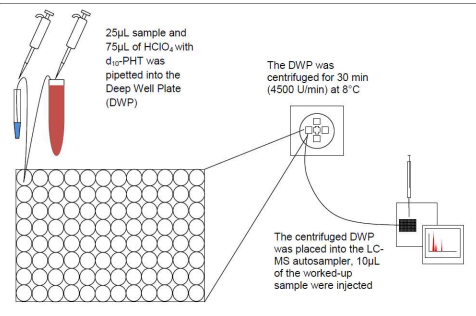
Fig 7: Quantitative comparison of LC-MS and GC-MS in arbitrary units.

## Introduction and Aim:

Phenytoin PHT is an anticonvulsant indicated for treatment and prevention of epilepsy. Little is known about the pharmacokinetics (PK) of PHT in the brain and the concentration correlation to samples used for therapeutic drug monitoring (TDM). This applies especially to the critical ill neurosurgical patient at high epileptic risk. As a prerequisite for PK investigations, a specific, sensitive, robust, and cost-effective analytical method in biological samples is mandatory. Therefore, two different, chromatographic methods (GC and LC) coupled with MS detection and the correlating sample preparation were compared. They were validated according to ISO 17025 /FDA Guidance for Industry. For better visualization of the comparison a scoring approach was aimed.



**Fig 1:** Clean-up of biological samples (aCSF, blood or saliva) for GC-MS analyses (SPE).



**Fig 2:** Sample preparation in deep well plates for LC-MS analyses for blood, saliva and aCSF samples.

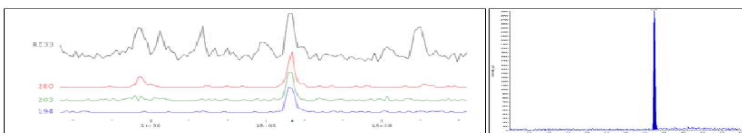
## Method:

**GC-MS:** capillary column: 0.25µm, DB-5ms, 0.25mm x 30m (Agilent). Temperature setting: 120°C-300°C (10°C/min). Injection volume: 2µL (Auto injector, Agilent 7673, 10µL syringe). Run time 25min. Spectra analysis MassLib™ (www.masslib.com) identification by similarity and identity. Calibration: 6 conc. levels from 50 to 1200 ng/mL were used and included the expected sample concentration from patients. A solid-phase extraction (SPE) with Bond Elute® LRC Certify 130MG Varian Inc. for clean-up of the samples (≥ 50µL) was used. The derivatization of the residue was made with trimethylsulphonium hydroxide. 5-(p-methylphenyl)-5-phenylhydantoin (MPPH) was used as internal standard (IS).

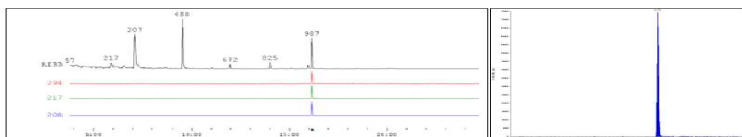
**LC-MS:** mobile phase A (H<sub>2</sub>O + HCOOH (100 + 0.1 v/v) and B (MeCN + HCOOH) were used. Pump1 with flow of 0.350 mL/min and pump2 with flow 0.200 to 1.000 mL/min (Dionex pump HPG-3200A). The trapping column Phenomenex Gemini Polar column, 2.0 x 10 mm, 5 µm was at room temperature. The main column Phenomenex Synergy Polar RP column, 2.0 x 50 mm was placed in the column oven (Cluzeau Info Labo CrocoCil) set on 50° C. The system was coupled to a MS (MDS Sciex, Q Trap 3200). d<sub>10</sub>-PHT (100 µg/mL in MeOH) was used as IS. This solution was diluted to 50 ng/mL with HClO<sub>4</sub>. 75 µL were used to deproteinize a sample of 25 µL. The calibration curve ranged from 10 to 2000 ng/mL.

**The validation** was made according to ISO/FDA Guidance for Industry including selectivity, sensitivity, recovery, limit of detection (LOD), limit of quantification (LOQ), accuracy, linearity, and extract stability analyses.

**The performance** of the methods was assessed by the sample volume needed, the time expenditure for an analysis in minutes, and the costs of materials in CHF. They were compared in arbitrary units.



**Fig 3:** GC-MS 50 ng/mL PHT (Cal1). Black = total ion chromatogram (TIC); red, green, and blue mass fragments at m/z 280, 203, 194



**Fig 4:** LC-MS 10 ng/mL (Cal1), PHT

**Fig 5:** GC-MS Internal standard 1200 ng/mL MPPH black = TIC; red, green, and blue mass fragments at m/z 294, 217, 208

**Fig 6:** LC-MS Internal standard 50 ng/mL d<sub>10</sub>-PHT

## Conclusions:

- The LC-MS method showed better performance (relative scoring 8.5 (Fig.8)).
- Less sample volume needed, reduced work and cost for sample preparation, and less chemicals needed.
- LC-MS has the, shorter run-time, and the larger linearity in the range from 10 to 2000 ng/ml covering the expected sample concentrations.

→ Therefore LC-MS is better suited for a extensive PK study.

## Results:

### GC-MS:

**Retention time:** PHT = 15.12min (Fig 3), MPPH = 16.15 (Fig 5).

**Selectivity and sensitivity:** all blank samples were negative.

**Recovery:** Quality control (QC) 100 ng/mL, QC 1000 ng/mL ≤ 8% min/max deviation from target value. **LOD** = 15 ng/mL, **LOQ** = 50 ng/mL for all matrices. **Accuracy:** 1-10% for calibrator 2 (Cal2) (150 ng/mL) to Cal6 (1200 ng/mL) and 20% for Cal1 (50 ng/mL). The calibration curve Cal1 to Cal6 was linear ( $r^2 \geq 0.995$ , aCSF \*n=8, blood \*n=2, and saliva \*n=2).

Dried extracts were stable ≥ 4 weeks (min/max deviation 4%).

Reinjection and storage (33h) on the auto sampler showed no variations.

**Run time:** 30 min. per analysis; the clean-up time for 25 samples took 5 h.

**Cost** for materials and consumables was about 8 CHF per sample.

### LC-MS:

**Retention time** for PHT and d<sub>10</sub>-PHT were at about 2.8 min. (Fig 4 and Fig 6).

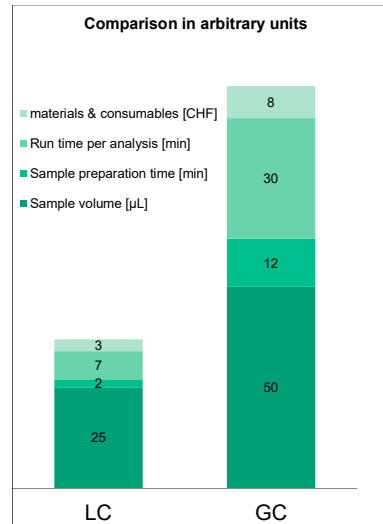
**Selectivity and sensitivity:** for blank samples no interferences were detected.

**Recovery:** min/max deviations from target value were 10% for the lowest QC and 3% for the highest QC. **LOD** <<10 ng/mL, **LOQ** = 10 ng/mL. **Accuracy:** 1-8% for Cal2 (20 ng/mL) to Cal8 (2000 ng/mL) and 3% for Cal1 (10 ng/mL).

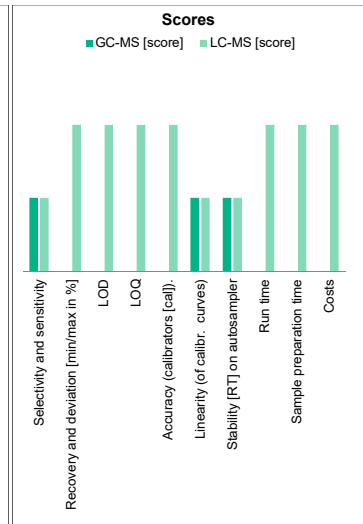
The calibration curve was linear ( $r^2 \geq 0.997$ , aCSF \*n=6, blood \*n=3, and saliva \*n=3). Reinjection after 7 days; no difference in accuracy was detectable. The **run time** is 7 min. per analysis, preparation time for 182 samples took 6 h.

(\*n = number of calibration curves)

Test	GC-MS [score]	LC-MS [score]
Selectivity and sensitivity	blank samples were negative [0.5]	blank samples were negative [0.5]
Recovery and deviation [min/max in %]	QC 100 ng/mL ≤8%, QC 1000 ng/mL ≤6%	QC 10 ng/mL 10% QC 1600 ng/mL 3% [1]
LOD	15 ng/mL	<<10 ng [1]
LOQ	50 ng/mL	10 ng [1]
Accuracy (calibrators [cal]).	1-10% for cal2 (150 ng/ml) to cal6 (1200 ng/mL) 20% for cal1 (50 ng/mL)	1-8% for cal2 (20 ng/mL) to cal8 (2000 ng/mL) 3% for cal1 (10 ng/mL) [1]
Linearity (of calibr. curves)	$r^2 \geq 0.995$ , aCSF n=8, blood n=2, and saliva n=2 [0.5]	$r^2 \geq 0.997$ , aCSF n= 6, blood n=3, and saliva n=3 [0.5]
Stability [RT on autosampler]	> 4 weeks (min/max deviation 4%). no effect after reinjection (33h) [0.5]	Reinjection after 7 days: no difference in accuracy [0.5]
Run time	30 Min	7 min. [1]
Sample preparation time	5h for 25 samples	6h for 182 samples [1]
Costs	8CHF	3CHF [1]
<b>Score</b> (winner got 1point)	<b>1.5</b>	<b>8.5</b>



**Fig 7:** Quantitative comparison of LC-MS and GC-MS in arbitrary units.



**Fig 8:** Score of the investigated parameters. Winner got 1 point if equal each got 0.5.

## Objectives

Phenytoin (PHT), an acidic lipophilic drug (m.w.=252.3, pKa=8.3, LogP=2.47), is routinely used to prevent and treat epilepsy. It has non-linear, individually variable pharmacokinetics, a small therapeutic index (C<sub>pl</sub>:10-20µg/ml or 40-80mM), and is 90% serum protein-bound; it needs therapeutic drug monitoring. The aims of this study were:

1. To define an appropriate sample extraction procedure for biological samples for GC-MS analysis of PHT.
2. To establish a GC-MS method suitable for PHT analysis in the samples and its validation according to ISO 17025.
3. To characterize the GC-MS method in order to compare it with an alternative LC-MS method to be developed.

## Materials and Methods

### Extraction :

For solid phase extraction (SPE) cartridges with non-polar C<sub>8</sub> sorbent and a strong cation exchanger (SCX) were used (Bond Elute® LRC Certify, 130MG, Varian Inc.).

Extraction procedure:

Conditioning: 1mL acetonitrile followed by 1mL citric buffer pH 5.

Probes with internal standard (IS) were added and washed: 1mL citric buffer pH 5 + 1mL HAc 0,01M to acidify (pH≈3.5), residue vacuum-dried for 5min. PHT eluted with 2mL acetone; 1min vacuum to drain the SPE columns. Transfer into GC analysis vials and dried to solid at 50°C (N<sub>2</sub>).

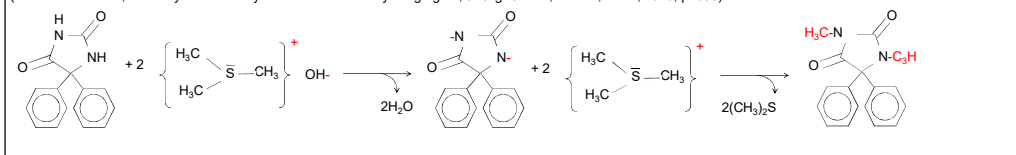
### GC-MS:

IS: 5-(p-methylphenyl)-5-phenylhydantoin (MPPH) m. w.=266.29, >99% purity (Sigma Aldrich)

Derivatization: Extracts were derivatized directly upon injection into the GC-MS with 50µL of TMSH (fig. 1)

Fig. 1: Derivatization of PHT with trimethylsulfonium hydroxide (TMSH)

(K. Yamauchi et al.; Trimethylsulfonium Hydroxide: A New Methylating Agent; J. Org. Chem., c'ol. 44, No. 4, 1979; p. 638)



GC: capillary column: 0.25µm, DB-5ms, 0.25mm x 30m (Agilent). Temperature: 120°C-300°C (10°C/min).

Injection volume: 2µL (Auto injector, Agilent 7673, 10µL syringe size). Run time 25min.

Spectra analysis: MassLib™ (www.masslib.com) identification by similarity and identity.

### Samples:

100µL microdialysate samples from ex situ brain tumor tissue (frozen by -20°C) were provided by the Kantonsspital Aarau, Switzerland.

Artificial cerebrospinal fluid (aCSF) samples, CPDA-preserved blood (Blood Donor Centre of Bern, Switzerland), and saliva (from investigator) were used and spiked with PHT(purity >96% Fluka, Switzerland), Na-PHT injection solution (Phenydan® Desitin Pharma, Switzerland), or CRS: PHT Ph.Eur.

### Chemicals:

Were of analytical grade (Merck, Fluka).

TMSH purchased from Macherey-Nagel

### Validation procedure:

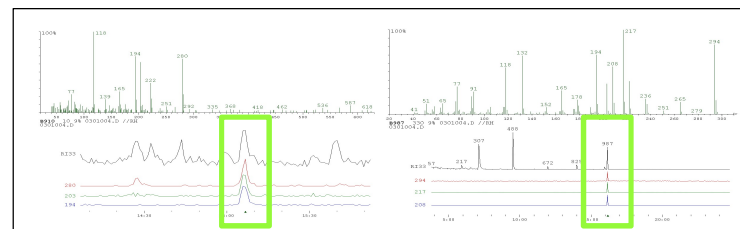
According to ISO 17025. 12 PHT calibration points at 6 conc. levels from 50 to 1200 ng/mL were used.

## Results (method characterization and validation):

**GC method** showed good PHT peak resolution, even in scan mode.

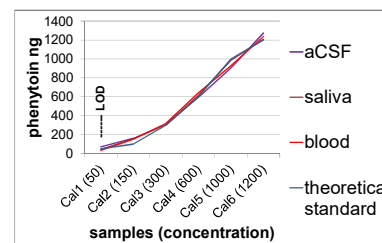
**IS:** MPPH was appropriate; ion fragments differ from PHT and are well separated (fig. 2).

Fig. 2: GC/MS Identification and Quantification: Retention time: PHT = 15.12 min, MPPH =16.15 min PHT 50ng/mL [280,203,194,118] and MPPH 1200ng/mL [294,203,194,118]



**Matrix effects:** One-way ANOVA analysis revealed no significant differences between aCSF, blood, and saliva: F= 0.0002, p=0.9998, (tab: 1), (fig: 3).

Fig. 3: Calibration curves for the matrices



Tab. 1: Correlation and linearity of PHT from different matrices: Results of spiked samples aCSF n=18; blood and saliva n=2 per conc.

Calibrator [ng/mL]	Mean <sub>aCSF</sub> +/-SD <sub>rel</sub> [%]	Mean <sub>blood</sub>	Mean <sub>saliva</sub>
C <sub>1</sub> 50	70 +/- 45	32 +/- 1	43 +/- 0.1
C <sub>2</sub> 150	161 +/- 10	150 +/- 17	153 +/- 6
C <sub>3</sub> 300	308 +/- 12	320 +/- 8	301 +/- 31
C <sub>4</sub> 600	594 +/- 7	640 +/- 1	614 +/- 3
C <sub>5</sub> 1000	905 +/- 10	929 +/- 13	984 +/- 3
C <sub>6</sub> 1200	1272 +/- 3	1237 +/- 36	1206 +/- 12

**Selectivity and sensitivity:** biological blank samples were negative, no false positives.

PHT limit of detection [LOD]: 50ng/mL (S/N ratio 4:1); PHT limit of quantification [LOQ]:100ng/mL

**Stability of the extracts:** dried extracts were stable >4 weeks at room temperature: QC1+/- 4%, QC2+/-1%. The stability of the dissolved extracts was >33 hrs (during stay in the auto sampler).

**Accuracy** (measured with calibrators between 50-1200ng/mL):

Inter-individual variability: +/-10%. Intra-individual variability: 1-32% (highest at LOD; 1-10% for the others).

**PHT recovery after SPE:** (spiked samples; 100% in absence of SPE):

94.1% for 100ng/mL, 94.32% for 1000ng/mL (n=3, r<sup>2</sup>=0.996).

## Conclusions

1. The GC-MS method is suited for PHT determination in biological samples of treated patients with appropriate sensitivity: 50-100ng representing 10% of the lower free serum value.
2. LOD & LOQ meet the requirements to monitor therapeutic dosing in liquor (≈100-200 ng/mL).
3. The GC-MS method needs a SPE, PHT derivatization, has a relatively long run time (≈30 min).
4. The SPE method can be used for all biological samples (dialysates, aCSF, blood, and saliva).
5. The validation meets analytical standards according to ISO 17025.

## Background and Aim of the study

Phenytoin (PHT), an acidic lipophilic drug, is used routinely in neurosurgical patients to prevent and treat epilepsy. The non-linear pharmacokinetics and the high variation of metabolism render a therapeutic drug monitoring (TDM) necessary. Data on PHT in CNS fluid of neurosurgical patients are lacking but should allow a better definition and monitoring of PHT pharmacokinetics at the site of action and compare them with other biological samples.

**The aim of this study was to define an appropriate sample extraction procedure and to establish a GC/MS method for PHT and its metabolites from brain microdialysates, blood, and saliva; the validation of the method was also targeted.**

## Materials and Methods

### Extraction and Derivatization:

Solid phase extraction cartridges (Bond Elute® LRC Certify; Varian) with acetonitrile-citrate buffer pH 5 (fig. 1). The acetone elute was dried to solid (50°C) under nitrogen. Dissolution and methylation with TMSH (trimethylsulfone hydroxide) prior to GC-MS analysis.

### GC-MS:

5-(p-methylphenyl)-5-phenylhydantoin (MPPH) was used as internal standard (IS).

The extracts were derivatized directly upon injection into the GC-MS. Oven temperature settings: 120°C – 280°C (10°C/min). Injection volume: 2µL (syringe size 10µL). Total run time (27min) showed good peak resolution (scan mode). MassLib™ (www.masslib.com) was used for spectra analysis (identification by similarity and identity).

### Samples:

Deep frozen (-20°C) 100 µL samples from ex situ brain tumor tissue microdialysis were provided by the Kantonsspital Aarau. Artificial (a)CSF was spiked for method evaluation and validation. CPDA-preserved blood was from the Blood Donor Centre of Bern. Saliva was provided by the first author.

### Chemicals:

Were of analytical grade (Merck, Fluka). Na-PHT injection solution (Phenydan® Desitin Pharma, Switzerland)). PHT (>96% Fluka, Switzerland), PHT CRS (Ph.Eur.). MPPH >99% purity (Sigma Aldrich).

### Validation procedure:

According to ISO 17025. PHT: 12 points calibration at 6 conc. levels (ranging from 50 to 1200 ng/mL).

Fig. 1: Sample solid phase extraction (SPE)

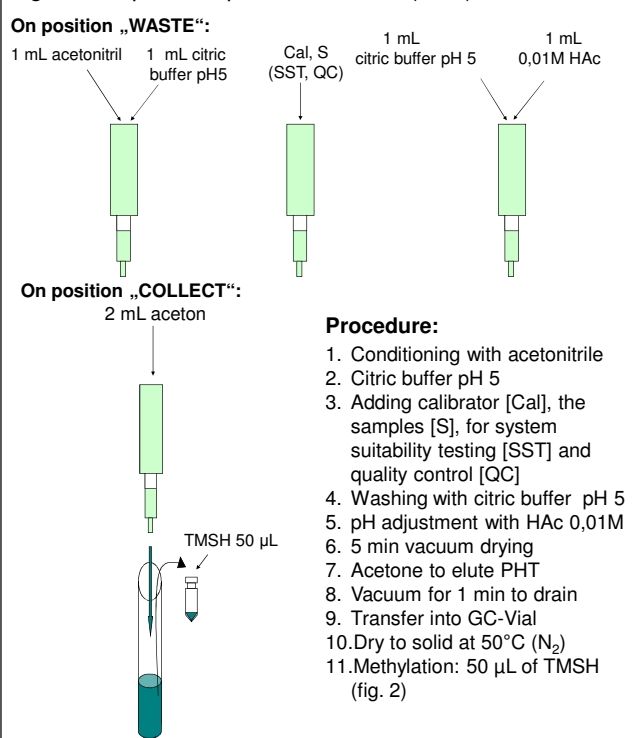
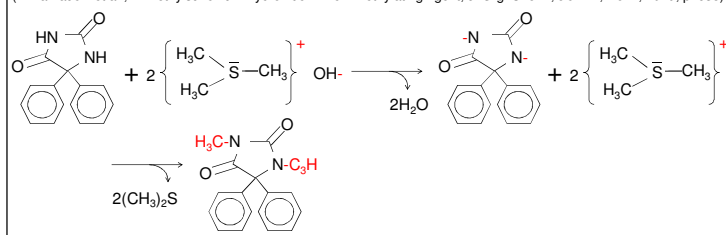


Fig. 2: Derivatization of PHT with trimethylsulfonium hydroxide (TMSH) (K. Yamauchi et al.; Trimethylsulfonium Hydroxide: A New Methylating Agent; J. Org. Chem., c'ol. 44, No. 4, 1979; p. 638)

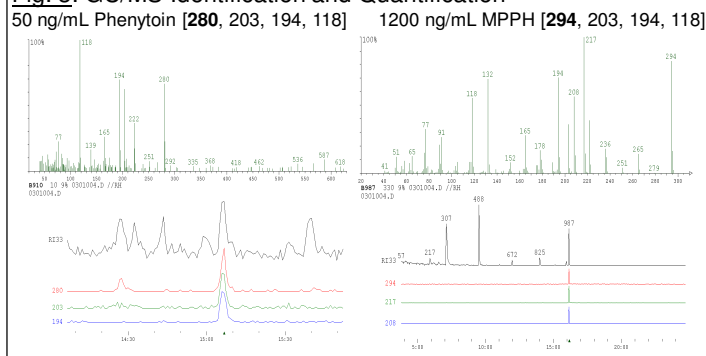


## Results

### Validation of the GC-MS-Analysis

IS: MPPH was an appropriate IS. Ion fragments differ from PHT and are well-separated. RT: PHT = 15.12 min, MPPH = 16.15 min (fig. 3).

Fig. 3: GC/MS Identification and Quantification



Tab. 1: Correlation and linearity of PHT from different matrices

Results of spiked samples aCSF n=18, blood and saliva n= 2 per conc.

Linear best fit		aCSF	blood	salvia
Correlation coefficient (r)		0.996	0.996	0.999
Slope (x) (calibration)		0.997	0.998	0.999
Axis intercept (y)		8.198	2.287	0.268
Calibrator [ng/mL]	Mean aCSF ± SD <sub>rel</sub> [%]	Mean <sub>blood</sub>	Mean <sub>salvia</sub>	
C <sub>1</sub>	50	70 ± 45	32 ± 1	43 ± 0.1
C <sub>2</sub>	150	161 ± 10	150 ± 17	153 ± 6
C <sub>3</sub>	300	308 ± 12	320 ± 8	301 ± 31
C <sub>4</sub>	600	594 ± 7	640 ± 1	614 ± 3
C <sub>5</sub>	1000	905 ± 10	929 ± 13	984 ± 3
C <sub>6</sub>	1200	1272 ± 3	1237 ± 36	1206 ± 12

For differences between aCSF, blood, and saliva series a oneway ANOVA analysis was calculated.

The results showed no significant differences (F= 0.0002; p=0.9998).

**Selectivity and sensitivity:** The selectivity was demonstrated (fig. 3). Biological blank samples were negative, no false positives were obtained. PHT limit of detection [LOD]: 50 ng/mL (S/N ratio 4:1) PHT limit of quantification [LOQ]: 100 ng/mL

**Stability of the extracts:** PHT in the dried extracts was stable > 4 weeks. The stability of the dissolved extracts (during stay in the auto sampler) was >33 hrs.

**Accuracy** (measured with calibrators between 50-1200 ng/mL):

Interindividual variability ± 10%.

Intraindividual variability: 1-32% (highest at LOD; 1-10% for the others).

**PHT Recovery after SPE** (spiked samples; 100% in absence of SPE): 94.1% for 100 ng/mL, 94.32% for 1000 ng/mL (n=3, r = 0.996)

## Conclusions

1. The method is suited for PHT determination from brain microdialysate, blood, and saliva in treated patients .
2. The validation meets analytical standards of ISO 17025.
3. LOD and LOQ meet the requirements to monitor therapeutic dosing in liquor (ca. 100-200 ng/mL).
4. The analytical procedure looks promising for the pharmacokinetic assessment of PHT in brain microdialysate and other human biological samples.

# Lagerung von Medikamenten in Lebensmittelkühlschränken: Eine günstige Alternative oder ein Sicherheitsrisiko?

Sabrina Grunert, Dr. T. Zysset und Raphael Hösli, Spitalzentrum Biel/Bienne

## Zusammenfassung

Das Europäische Arzneibuch definiert die Lagerung von Medikamenten "im Kühlschrank" zwischen 2°C bis 8°C. Auf älteren Stationsapotheken des Spitalzentrums Biel werden dafür oft noch Lebensmittelkühlschränke verwendet. Ob diese für die Medikamentenlagerung geeignet sind wurde im Hinblick auf einen anstehenden Umbau der Stationszimmer im SZB an zwei unterschiedlich grosse Lebensmittelkühlschränken der Firma Liebherr Kühlgeräte evaluiert. Es zeigte sich, dass eine korrekte Lagerung nach Ph. Eur. nicht möglich ist.

## Hintergrund und Ziel

Das Europäische Arzneibuch definiert die Lagerung von Medikamenten "im Kühlschrank" zwischen 2°C bis 8°C. Auf älteren Stationsapotheken werden dafür oft noch Lebensmittelkühlschränke verwendet. Ob diese für die Medikamentenlagerung geeignet sind wurde im Hinblick auf einen anstehenden Umbau der Stationszimmer im SZB an diversen Kühlgeräten evaluiert.

## Methode

An 2 unterschiedlich grossen Lebensmittelkühlschränken der Firma Liebherr (ein Standmodell (263L Inhalt) und ein kleineres Einbaumodell (123L Inhalt)), wurden folgende Messungen durchgeführt:

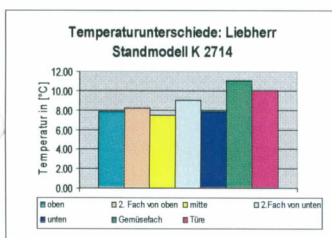
- Erfassung der Temperaturgradienten, insbesondere in den kritischen Zonen wie Türinnenseiten und Gemüsefach
- Messung des Temperaturverlaufes bei Stromausfall bei geschlossenem Kühlschrank
- Messung der Erwärmung von flüssigen und festen Medikamenten nach Entnahme aus dem Kühlschrank.

Zur Temperaturmessung dienten digitale Thermometer der Marke "Digital Thermo in/out" von Migros, welche mit einem geeichten Flüssigkeitsglasthermometer validiert wurden. Mit einer Stoppuhr wurden die zeitlichen Verläufe der Temperatur in den geschlossenen Geräten aufgezeichnet. Auf jedem Niveau wurde die Temperatur dreimal gemessen. Zum Vergleich wurden die Temperaturzonen eines Medikamentenkühlschranks (325L Inhalt) der Firma Liebherr Mediline erfasst.

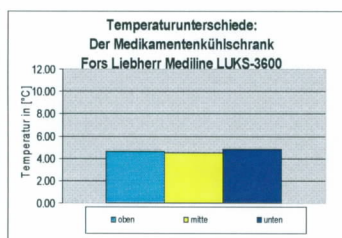
## Resultate

### Temperaturgradienten in verschiedenen Kühlgeräten

Die Temperaturunterschiede in den Lebensmittelkühlschränken schwanken zwischen 7.4-11°C (Grafik 1). Im Vergleich dazu sind Temperaturschwankungen im Medikamentenkühlschrank kaum vorhanden (4.1-4.9°C) (Grafik 2).



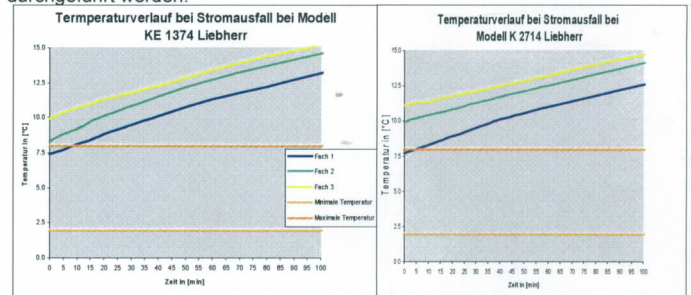
Grafik 1: Temperaturen an verschiedenen Stellen in einem Lebensmittelkühlschrank



Grafik 2: Temperaturen an drei Stellen in einem Medikamentenkühlschrank Propeller zur Luftzirkulation

### Temperaturverlauf bei einem Stromausfall

Bereits 10 min nach Stromausfall kommt es zum Überschreiten der Solltemperatur (2°C bis 8°C, Grafiken 3 und 4). Der Vergleich mit einem Medikamentenkühlschrank konnte aus Logistischen- und Kostengründen nicht durchgeführt werden.



Grafik 3: Temperaturverlauf bei Stromausfall bei einem Volumen von 123L

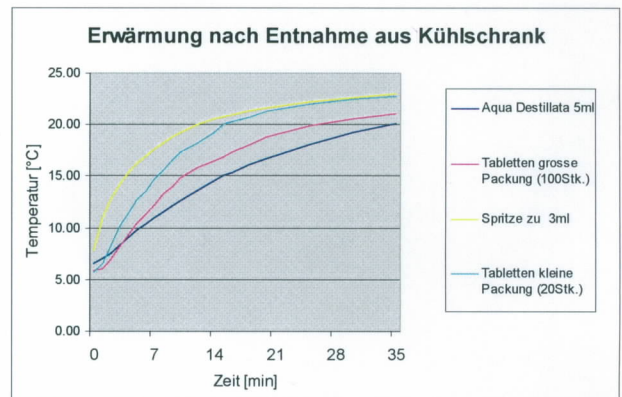
Grafik 4: Temperaturverlauf bei einem Stromausfall und einem Volumen von 263L

### Temperaturverlauf verschiedener galenischer Formen nach Entnahme aus dem Kühlschrank

- Je nach Ausgangstemperatur und galenischer Form dauerte es nur ein bis drei Minuten bis die Temperatur des Medikaments auf über 8°C gestiegen ist.
- Die Zeit bis zur Überschreitung von 20°C ist hängt stark von der galenischen Form ab:

*Inneres einer Tablettenschachtel:* 5 Minuten

*Flüssigkeiten:* Bis 90 Minuten.



Grafik 5: Erwärmung von verschiedenen Arzneiformen und Packungsgrößen nach der Entnahme aus dem Kühlschrank

## Diskussion

- Lebensmittelkühlschränke sind zur Lagerung von Medikamenten ungeeignet. Der Temperaturunterschied innerhalb des Kühlgerätes und die zugelassenen Tagesschwankungen sind zu gross um eine korrekte Lagerung zu garantieren.
- Die Zeit nach einem Stromausfall, bis eine Temperatur über 8°C erreicht wird, ist sehr kurz. Es muss sofort reagiert werden und ein grosser Aufwand ist nötig um die Kühlkette garantieren zu können.
- Der Transport von Kühlware hat ausschliesslich in Kühlboxen mit Kühlelementen zu erfolgen. Bei einem ungekühlten Transport auf eine Station kommt es rasch zum Übersteigen der zulässigen Temperatur.

## Ausblick

Die Kosten für neue Medikamentenkühlschränke stehen in keinem Verhältnis zur Gefahr, welche durch unkorrekte Lagerung von Medikamenten entsteht. Daher ist es unabdingbar Medikamente nur in dafür deklarierten Medikamentenkühlschränken zu lagern. Bei einem Stromausfall müssen die Medikamente sofort in einer Kühlbox in ein anderes Kühlgerät gebracht werden. Aufgrund dieser Untersuchungen wurden nun am SZB für alle Stationen Medikamentenkühlschränke bewilligt.



## Background and Aim of the study

Phenytoin (PHT) is an acidic lipophilic drug and is used routinely on neurosurgical (ICU) patients to prevent and treat epilepsy. The non-linear pharmacokinetics and the high variation of metabolism render a therapeutic drug monitoring (TDM) necessary. Extracellular CNS fluid available from continuous microdialysis in ICU patients should allow a better definition and monitoring of PHT pharmacokinetics at the site of action. A GC-MS method was selected to monitor the parent compound in small CNS microdialysis samples.

The aim of the study was to define an appropriate sample extraction procedure and to demonstrate the suitability of the analytical method by validation according to ISO 17025.

## Methods

**Extraction:** Solid phase extraction cartridges (Bond Elute® LRC Certify; Varian) were used with acetonitrile-citrate buffer pH 5 (see fig. 1). The acetone-eluate was dried to solid (50°C) in a stream of nitrogen, dissolved and methylated with TMSH (Trimethylsulfone hydroxide) prior to GC-MS analysis.

**GC-MS:** 5-(p-Methylphenyl)-5-Phenylhydantoin (MPPH, > 99% purity, Sigma Aldrich) was used as internal standard. The 50 µL extracts were derivatized directly upon injection into the GC-MS. During the total run time of 27 min., good peak resolution (scan mode) was obtained. Settings: Temperature start 120°C, to a final temperature of 280°C with 10°C/min. All chromatographic peaks were checked with MassLib™ (www.masslib.com) allowing mass spectra identification by both similarity and identity.

**Samples:** 200-500 µL biological samples were used, provided from the Kantonsspital Aarau (Prof. Hans Landolt, Head neurosurgery) and stored deep frozen (-24°C). Artificial CSF was spiked for method evaluation and validation.

**Chemicals:** All of analytical grade (Merck, Fluka). Na-PHT injection solution (Phenyhdan® Desitin Pharma, Switzerland), PHT (>96% Fluka), PHT CRS (Ph.Eur.)

**Validation procedure:** According to ISO 17025. A 12-point calibration for PHT at 6 different calibration levels, from 50 to 1200 ng/mL was used.

Fig 1: Sample extraction procedure

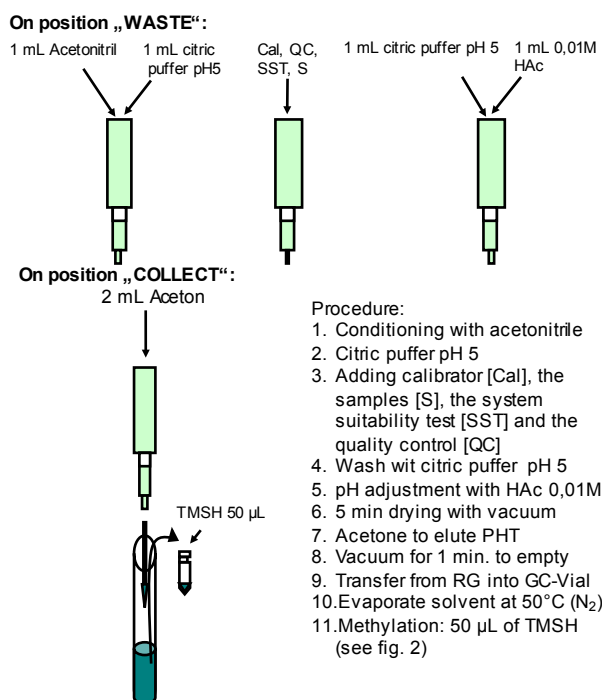
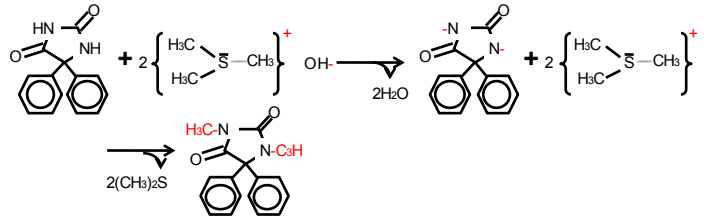


Fig 2: Derivatisation of PHT with trimethylsulfonium hydroxide [TMSH].

[K. Yamauchi et al.; Trimethylsulfonium Hydroxide: A New Methylating Agent. J. Org. Chem., c'ol. 44, No. 4, 1979; p. 638]



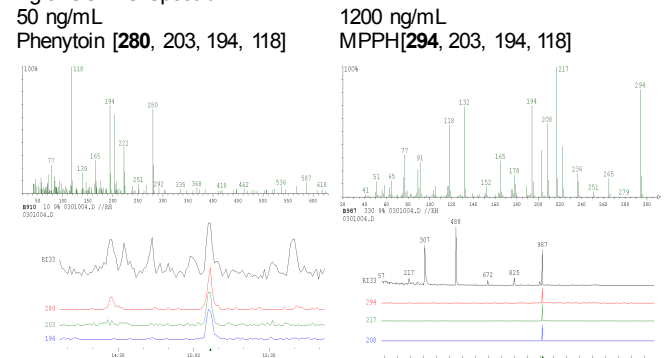
## Results

### Validation of the GC-MS-Analysis

#### Internal standard

MPPH was appropriate as internal standard. PHT and MPPH have different Ion fragments and could therefore easily be separated. The retention time differs by 1 minute (RT: PHT = 15.12 min., MPPH = 16.15 min).

Fig 3: GC-MS-Spectrum



### Correlation and linearity of PHT

Correlation coefficient between 50 and 1200 ng/mL:  $r^2 = 0.993$ .  
 Deviation from the target for calibrators: 40% [50 ng/mL], 7% [150 ng/mL], 3% [300 ng/mL], 1% [600 ng/mL], 9% [1000 ng/mL], 6% [1200 ng/mL].

### Selectivity and sensitivity

The selectivity was demonstrated. The blank biological samples were negative, so no false positives were obtained.

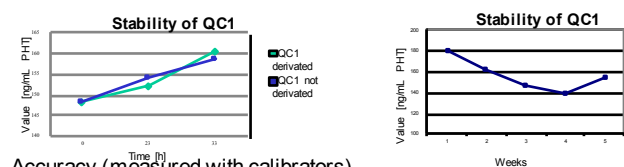
Limit of detection [LOD]: 50 ng/mL (S/N-ratio 4:1) PHT

Limit of quantification [LOQ]: 100 ng/mL PHT

### Stability of the extracts

PHT in the dried extracts was stable > 4 weeks. The stability of the dissolved extracts (during analysis) is > 33 hrs.

Fig 4: Stability of QC1



### Accuracy (measured with calibrators)

Interindividual variability +/- 10%.

Intraindividual variability: 1 - 32% (highest at LOD; 1 -10% for the others).

### Recovery (spiked samples with and without SPE)

The amount of PHT after SPE was 89% for 100 ng/mL, 93% for 1000 ng/mL and 45 -115% for 50 ng/mL (LOD) compared without SPE.

## Conclusions

1. The method is suitable for PHT determination in extracellular CNS fluid.
2. The validation meets analytical standards of ISO 17025.
3. LOD and LOQ are sufficient to monitor therapeutic dosing, for liquor ca. 100-200 ng/mL.
4. The analytical procedure looks promising for the pharmacokinetic assessment of PHT in brain.