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Article

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

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

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

Introduction

Epilepsy is a disorder of the central neural system characterized by recurrent unprovoked seizures caused by excessive discharge of electrical activity (1). Epilepsy can be treated with different antiepileptic drugs that generally render 80% of newly diagnosed patients seizure-free (2). Phenytoin (PHT) [$C_{15}H_{12}N_2O_2$ (3); molecular weight: 252.3 (3)] is a well-established antiepileptic drug designed to prevent and treat seizures (4). It is routinely used on neurosurgical intensive care unit patients with brain injuries.

PHT has a small therapeutic index; its therapeutic concentration range in blood serum is 10–20 mg/L (40–80 μmol/L) (5) for adults and children older than 3 months. Ingestion of more than 20 mg/kg in humans [normal oral dosage for adults is 6 mg/kg (5)] usually results in clinical toxicity (6). PHT can produce significant dose-related toxicity because of its complex pharmacokinetics (PK). The limited therapeutic index, combined with the large inter-individual variability of metabolism [half life 16–60 h, depending on plasma levels (7, 8)], and the nonlinear pharmacokinetics of PHT (9, 10) highlight the importance of therapeutic drug monitoring (TDM). On the other hand, relatively few studies have been able to demonstrate the benefits of TDM of antiepileptic drugs (11). However, therapeutic monitoring of PHT using Bayesian forecasting was successfully applied to rapidly achieve therapeutic plasma levels using an easy-to-apply PHT loading dose regimen in a hospital setting (12).

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

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

Commonly used analytical methods without mass spectrometry (MS) detection do not reach the sensitivity needed to 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 μL used in a recently published study is far too large for the usual microdialysis sampling in patients, with a 2 μL/min flow rate, and therefore not appropriate for TDM (21). GC–MS analysis needs an extraction step such as solid-phase extraction (SPE) to clean the sample and to eliminate interfering biological matrix materials like proteins and lipids prior to injection into the GC–MS system. Furthermore, to detect PHT with sufficient sensitivity by GC–MS, a derivatization of PHT is necessary (22, 23). A structural analogue to PHT ($C_{16}H_{14}N_2O_2$; molecular weight: 266.3), 5-(*p*-methylphenyl)-5-phenylhydantoin (MPPH), is chosen as an internal standard (IS) (24).

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

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

Material and Methods

Chemicals and samples

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

Artificial cerebrospinal fluid (aCSF), composed of 8.59 g NaCl, 0.2013 g KCl, 0.1332 g $CaCl_2$ and 0.1758 g $MgCl_2 \times 6(H_2O)$, was prepared according to the pre-clinical device company CMA (Stockholm, Sweden; dialysate solution) (25). CPDA-1 blood (an anticoagulant citrate phosphate dextrose adenine solution; subsequently referred to as blood plasma) was obtained from the Blood Donor Center in Bern, Switzerland. Saliva was provided by a volunteer. *Ex situ* brain tumor tissue, which had been surgically removed from patients treated with PHT, was dialyzed against aCSF with a flow rate of 2 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

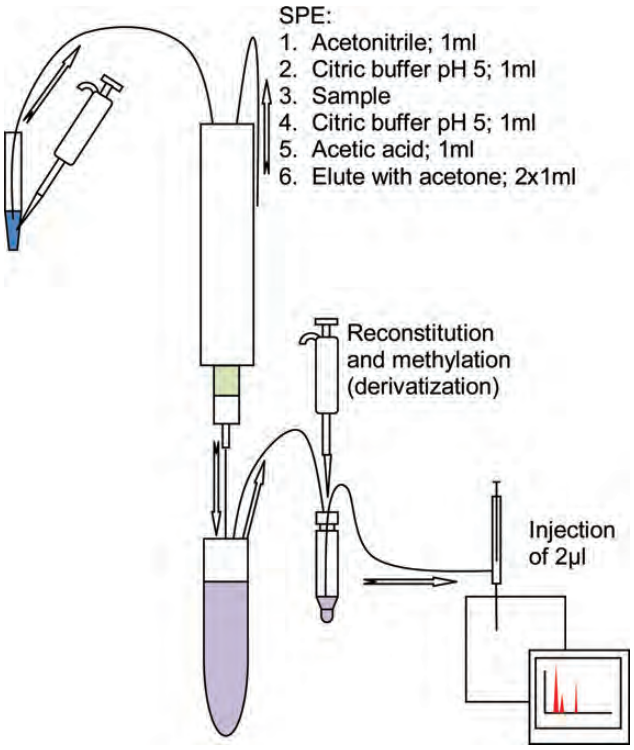

Figure 1. Schematic sample process: generation of the three sample matrices, processing during SPE and derivatization prior to GC–MS measurement.

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

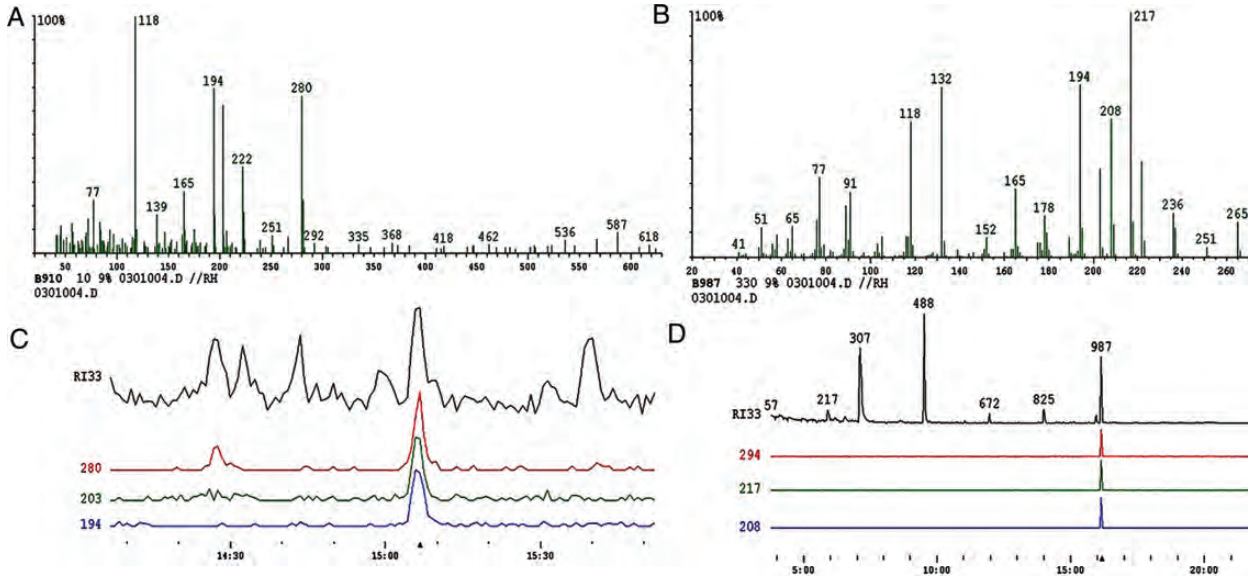

Figure 2. Total ion chromatograms (TICs) and MS fragment spectra of PHT (50 ng/mL) and MPPH (1,200 ng/mL). GC–MS chromatograms: (A) PHT; (B) IS MPPH; (C) MS fragments and a 2.0 mL injection of a 50 ng/mL PHT solution LOQ; (D) time-related peaks and 1,200 ng/mL MPPH. (A) The ion fragments for PHT are [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 I). The calibrator values showed minimum and maximum percent deviations of 20% in Cal 1, 7% in Cal 2, 3% in Cal 3, 1% in Cal 4, 9% in Cal 5, 6% in Cal 6, 12% in QC1 and 6% in QC2 (Table I). Statistically, the deviations of each concentration value did not differ for the entire calibration, demonstrating that there were no outliers. All of the values were within the maximum allowed bias of 15% (32). Repeatability precision (inter-assay variability), the minimum and maximum deviation of all levels of measurements, was within 15% of the coefficient of variation (CV) (Table I).

Regarding laboratory precision (intra-assay variability), for the QC 1 and QC 2 measurements ($n = 6$), the mean percent deviation from the target value was 2.7% for QC 1 (100 ng/mL) and 0.6% for QC 2 (1,000 ng/mL). As with the calibrators, the QC 1 and QC 2 also showed a deviation from the nominal value of less than 15% CV. These results support the robustness of the method. Neither the operator nor the day of preparation influenced the results in a detectable manner. There was no difference in the characteristics of the chromatogram when changing from the GC–MS HP system to the backup (Agilent Systems). The laboratory in which the investigations were made is part of the forensic laboratories in Switzerland and takes part in all necessary proficiency tests to be ISO 17025 certified.

Linearity and regression coefficient of calibration curve

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

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

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

Calculated data from the measurements with aCSF as matrix

Sample name	Overall mean	SD	Mean deviation (%)	Inter-assay CV (%)	Accuracy (%)
Cal 1 (50 ng/mL)	52.3	6	9.65	11.5	104.6
Cal 2 (150 ng/mL)	152.5	16.9	7.51	11.1	101.7
Cal 3 (300 ng/mL)	296.7	33.3	9.33	11.2	98.9
Cal 4 (600 ng/mL)	597	55	6.75	9.2	99.5
Cal 5 (1,000 ng/mL)	996	48	3.55	4.8	99.6
Cal 6 (1,200 ng/mL)	1,220	65	4.46	5.3	101.7
QC 1 (100 ng/mL)	102.7	6	2.7	6.3	102.7
QC 2 (1,000 ng/mL)	1,005.7	35	0.6	3.6	100.6

Arithmetic mean of the calibrators in different matrices

Calibrator (concentration)	aCSF mean/deviation from target concentration ($n = 6$)	Saliva mean/deviation from target concentration ($n = 3$)	Blood plasma mean deviation from target concentration ($n = 3$)
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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