



Influence of ultrafiltration conditions on the unbound fraction of phenytoin and valproic acid

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Abstract

Purpose This study was designed to analyse the influence of temperature, pH and storage time on unbound fractions of PHT and VPA.

Methods The influence of ultrafiltration (UF) temperature on measured unbound fractions of PHT and VPA in spiked samples was evaluated in a single laboratory experiment and in data from a national external quality control (EQC) database. The influence of pH adjustment with phosphate buffered saline (PBS) on measured unbound fractions of PHT and VPA was investigated in patient samples. The influence of storage time on unbound fractions of PHT and VPA was examined in patient samples by performing UF at various time points.

Results Performing UF at a temperature of 37 °C compared to 20 °C significantly increased ($p < 0.05$) the measured unbound fractions for both PHT (range 14.8–26.3%) and VPA (range 5.7–16.0%) in the single laboratory experiment. Consistent with these findings, reported unbound fractions in the EQC database of PHT were significantly higher with UF at 37 °C compared to room temperature. For VPA, this was not the case. However, for both drugs, the number of laboratories performing UF at 37 °C was limited ($n = 2–4$). Adjustment of pH with PBS was unfeasible. After 4 days of storage at 5 °C, the unbound fraction of PHT seemed to remain stable whereas the unbound fraction of VPA appeared to increase (27.4%) ($n = 3$).

Conclusion Higher UF temperatures led to an increase in measured unbound fractions of both PHT and to a lesser extent VPA. Storage time of plasma samples might influence the unbound fraction of VPA.

Keywords Phenytoin · Valproic acid · Unbound fraction · Ultrafiltration conditions

Introduction

Phenytoin (PHT) and valproic acid (VPA) are widely used and effective antiepileptic drugs, while VPA is also indicated for the treatment of bipolar disorder [1, 2]. For both drugs, therapeutic drug monitoring (TDM) is used to optimise therapy. TDM is particularly applied in cases of insufficient efficacy, drug-drug interactions, impaired renal function or in specific populations such as children or pregnant women [3]. Both the efficacy and the toxicity of PHT and VPA are attributed to their free, unbound concentrations [4]. However, due to the higher cost and more complex analysis of unbound concentrations, most clinical laboratories measure total concentrations. Unbound concentrations can subsequently be estimated using models based on protein binding [5, 6]. However, for both PHT and VPA, it has been shown that model-based estimated unbound concentrations often inaccurately represent the actual situation. This is probably

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explained by the relatively high and variable protein binding of both drugs [7–9]. Therefore, it is advised to measure unbound concentrations instead.

When measuring unbound concentrations, it is important to take into consideration that ultrafiltration (UF) conditions may differ from physiological conditions, possibly resulting in an inaccurate representation of unbound concentrations. For example, due to pH increase while storing samples, pH in plasma samples might differ from physiological pH, possibly influencing measured unbound concentrations [10]. Furthermore, UF is often conducted at temperatures below the physiological body temperature of 37 °C, which might influence the degree of protein binding to the drug and thus affect the measured unbound concentrations [10, 11].

Moreover, the storage time of plasma samples (i.e. time before UF) can influence the unbound fraction. It has been suggested that after long-term storage of plasma samples, plasma lipolysis occurs that can result in fatty acid-induced conformational changes [12]. These changes may alter the binding of drugs to proteins, consequently affecting the measured unbound fractions.

To ensure adequate and uniform measurement of unbound concentrations, this study was designed to analyse the influence of UF temperature, pH and storage time on unbound fractions of PHT and VPA.

Materials and methods

Study design

A single laboratory experiment was conducted and a national external quality control database (EQC) was used to evaluate the influence of UF temperature on the measured unbound fractions of PHT and VPA. Furthermore, the influence of pH on the measured unbound fractions of PHT and VPA was investigated. Lastly, the influence of plasma sample storage time on PHT and VPA concentrations was analysed by measuring total and unbound concentrations at various timepoints.

Patient samples collected for routine TDM of PHT or VPA were used from patients that provided general consent for the use of their data for research purposes upon admission to the hospital. Patient samples and data were processed anonymously.

Influence of UF temperature

Single laboratory experiment

For PHT, samples in three concentration levels were prepared based on the therapeutic reference range of total plasma concentrations (10–20 mg/L), i.e. 6 mg/L (low),

12 mg/L (mid) and 24 mg/L (high), each in five-fold [3]. Similarly, three concentration levels were prepared for VPA based on the therapeutic reference range of total plasma concentrations (50–100 mg/L), i.e. 40 mg/L (low), 80 mg/L (mid) and 160 mg/L (high) [1]. All samples were prepared in Omniplasma® (pooled, solvent/detergent, prion reduced human plasma). Per concentration level and UF temperature (10 °C, 20 °C, 25 °C and 37 °C), samples were prepared and measured in five-fold.

National EQC database

An EQC programme is organised in the Netherlands by the Drug Analysis and Toxicology Division (KKG) of the Dutch Foundation for Quality Assessment in Medical Laboratories (SKML, <https://www.skml.nl/en/sections/drug-analysis-and-toxicology>). This programme is organised for various drugs, including PHT and VPA. As part of the programme, KKG distributes spiked samples (human freeze-dried serum) to participating laboratories, which then analyse the samples and submit their findings. SKML collects the data, provides feedback and assigns performance scores. Reported data on PHT and VPA from all EQC rounds in 2023 (2023.1, 2023.2, 2023.3, 2023.4) and the first round of 2024 (2024.1), including measured unbound fractions and UF temperature, was retrieved from the database. The samples were part of a dedicated round of the EQC programme focused on unbound antiepileptic drug concentrations and, as such, only contained PHT and VPA.

Influence of sample pH during UF

For both drugs, two patient samples previously obtained for routine TDM were used to assess the influence of sample pH during UF. Plasma was collected in BD-Vacutainer Clot activator tubes®. Patient samples were stored in the refrigerator (5 °C) until routine TDM occurred. After TDM had been employed and pH was measured (within 24 h of sample collection), half of the plasma sample was stored in the refrigerator (5 °C) while the other half was stored frozen (–20 °C). This was done to account for potential pH variations as a result of the storage method used. After 2 weeks of storage, the sample pH and unbound fractions of PHT and VPA were measured. Subsequently, attempts were made to adjust the sample pH to 7.4, reflecting in vivo conditions, by diluting the samples with phosphate buffered saline (PBS) (J.T.Baker®, pH 7.15–7.25) at a 1:5 ratio. Two aliquots of every sample were taken. One was ultrafiltered at 25 °C and the other at 37 °C. At both UF temperatures, unbound fractions of PHT and VPA were measured. The pH of samples was measured using litmus paper.

Influence of storage time

For both drugs, three patient samples previously obtained for routine TDM were used for measurement of unbound fractions to determine the influence of storage time. Samples were stored at 5 °C. UF was performed at a temperature of 25 °C at four different timepoints: day 0 (immediately after collection), day 1, day 3 (PHT) or day 4 (VPA) and day 7. Ultrafiltrates were stored at 5 °C and analysed collectively on day 7. The different timepoints for PHT and VPA were selected based on sample collection timing and weekend scheduling.

Bioanalytical measurement of total and unbound PHT and VPA concentrations

Total and unbound concentrations of PHT and VPA were analysed using a validated UPLC-MS/MS method. For the total concentration, sample treatment consisted of protein precipitation with a methanol-based solution containing stable isotopically labelled internal standards, including PHT-D10 and VPA-D6. To obtain the unbound fraction of PHT and VPA, 500 µl plasma was brought onto an Amicon Ultra 4 (30 k) centrifugal filter device (Millipore). The filters were not pre-washed. Subsequently, UF took place by centrifugation for 15 min at 5088 g (relative centrifugal force) in a pre-equilibrated centrifuge (centrifuge: Sorvall ST 16 R, Thermo Scientific, Bleiswijk, The Netherlands) at the desired temperature. The UF samples (50 µl) were spiked with the internal standard (200 µl), containing PHT-D10 and VPA-D6. All samples were centrifuged for 6 min at 14,620 g after which the supernatant (50 µl) was diluted with water (1000 µl) and injected into the UPLC column. The chromatographic separation was performed using a Waters column (ACQUITY UPLC BEH C18, 1.7 µm; 2.1 mm × 50 mm, Etten-Leur, The Netherlands), at a flow rate of 0.4 ml/min, with a gradient of ammonium acetate in H₂O/MeOH (90/10 v/v) and 100% MeOH. Analytes were measured in positive (PHT) or negative (VPA) electrospray ionisation mode using multiple reaction monitoring (MRM). The capillary voltage was set to 1.50 kV with a source temperature of 150 °C and a desolvation temperature of 500 °C. The desolvation gas flow was 900 L/h (nitrogen) and the cone gas flow was 25 L/h (nitrogen). Nitrogen was used as the nebuliser gas, while argon served as the collision gas. MRM transition, cone voltage (PHT 26 V, VPA 32 V) and collision energy (PHT 36 V, VPA 5 V) were optimised using IntelliStart (Waters). The linear measurement range was 0.25–20 mg/L for PHT and 1.25–200 mg/L for VPA. Recovery in validation experiments averaged 97.0% for PHT and 97.9% for VPA in EQC samples and 103.9% for PHT and 98.2% for VPA in spiked samples. The relative standard deviation in EQC samples was 3.7% for PHT and 2.4% for VPA and in spiked samples 10.1% for PHT and 6.4% for VPA.

Statistical analysis

Unbound fractions of PHT and VPA were calculated as percentages by dividing the measured unbound concentration (in ultrafiltrate) by the measured total concentration (in plasma). Both total and unbound concentrations were measured for each sample to obtain the measured unbound fraction. Differences between the measured unbound fractions in the experiments with spiked samples were calculated with analysis of variance (ANOVA). When the ANOVA test was statistically significant, post hoc analysis was performed using Tukey's method or Games-Howell if equal variances were not assumed. Differences between the reported unbound fractions from the national EQC programme were calculated using a non-parametric Mann-Whitney test. The influence of storage time on unbound fractions was analysed using a non-parametric Friedman test. SPSS version 24 was used.

Results

Influence of UF temperature

Single laboratory experiment

Measured unbound fractions of both PHT and VPA increased when centrifuged at higher UF temperatures. Results are shown in Fig. 1a (PHT) and Fig. 1b (VPA). There was a statistically significant ($p < 0.05$) difference between all measured unbound fractions in both PHT and VPA, with the exemption of 20 °C vs. 25 °C at a total concentration of 24 mg/L (high) for PHT and 160 mg/L (high) for VPA. When comparing results of the measured unbound fractions of 20 °C to 37 °C for PHT, measured unbound fractions increased by 26.0%, 26.3% and 14.8% for total concentrations of 6 mg/L, 12 mg/L and 24 mg/L, respectively. When comparing results of the measured unbound fractions at an UF temperature of 20 to 37 °C for VPA, measured unbound fractions increased by 16.0%, 14.2% and 5.7% for total concentrations of 40 mg/L, 80 mg/L and 160 mg/L, respectively.

National EQC database

In the different EQC rounds, 21 to 26 laboratories participated and reported the used UF temperature, which in most (18 or more, varying per round) laboratories was room temperature (reported as 20 to 25 °C). UF at physiological temperature (37 °C) was only deployed at two to four (varying per round) laboratories for PHT, while for VPA, this was deployed at only three laboratories. None of the laboratories reported measuring the pH. Rounds 2023.1–2024.1 were spiked with a total concentration of PHT of 14.8 mg/L, 15.3 mg/L, 15.4 mg/L, 15.0 mg/L and 9.9 mg/L, respectively.

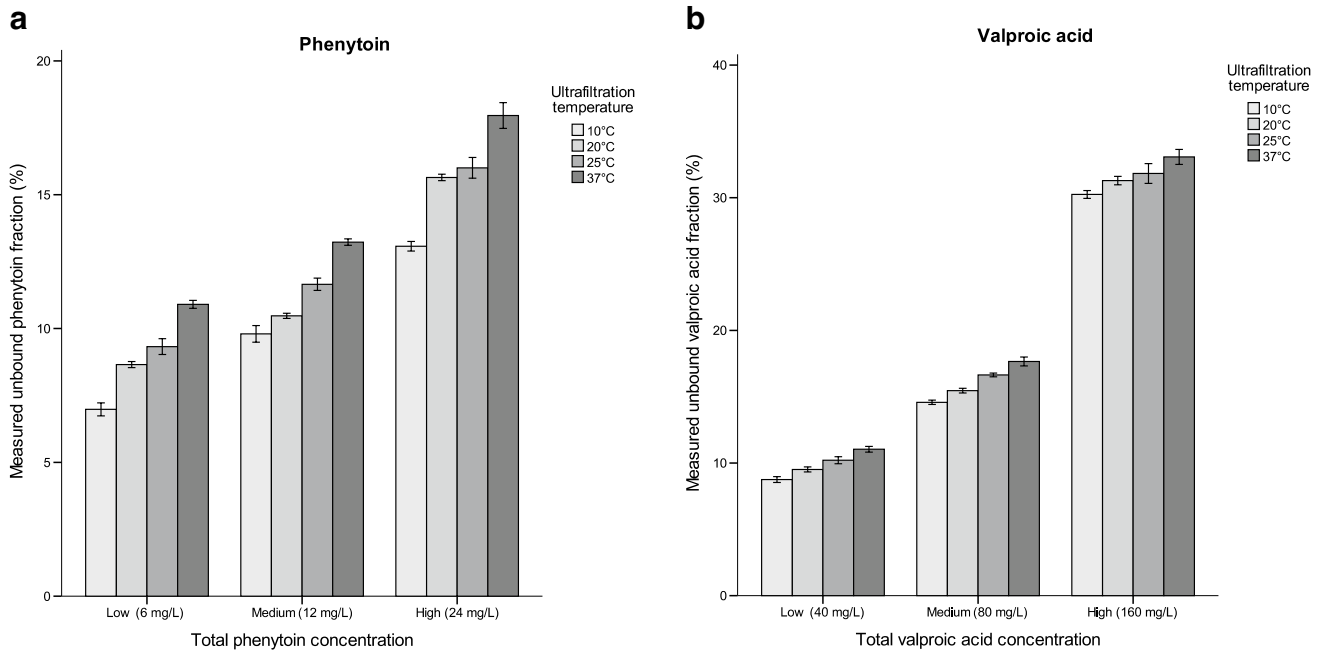


Fig. 1 **a** Measured mean unbound fractions (%) of phenytoin (PHT) measured using four different ultrafiltration (UF) temperatures (10°C, 20°C, 25°C and 37°C) for three different spiked total PHT concentration levels. N=5 for each UF temperature and concentration level. Results are shown as mean± standard deviation. **b** Measured mean

unbound fractions (%) of valproic acid (VPA) measured using four different ultrafiltration (UF) temperatures (10°C, 20°C, 25°C and 37°C) for three different spiked total VPA concentration levels. N=5 for each UF temperature and concentration level. Results are shown as mean± standard deviation

For VPA, rounds 2023.1–2024.1 were spiked with a total concentration of 99.6 mg/L, 102.6 mg/L, 103.8 mg/L, 100.7 mg/L and 69.1 mg/L, respectively.

For PHT, there was a statistically significant increase in reported unbound fractions at an UF of 37 °C compared to room temperature in rounds 2023.2, 2023.3, 2023.4 and 2024.1 ($p < 0.05$). For VPA, there was no statistically significant difference in reported unbound fractions between UF at 37 °C and UF at room temperature. Results are shown in Fig. 2a (PHT) and Fig. 2b (VPA).

Influence of sample pH during UF

The average pH of the patient samples, measured within 24 h after sample collection, was 8.0 (range 7.9–8.1). After storage for 2 weeks, the average pH increased to 8.4 (range 8.3–8.5). Subsequently, after diluting the samples with PBS in a ratio of 1:5, the pH decreased to 7.8 (range 7.7–7.9). There were no differences in pH between samples stored at 5 °C or –20 °C, nor between both drugs. Total concentrations of PHT decreased by 2.4% after storage for 2 weeks at 5 °C and increased by 0.5% after 2 weeks of storage at –20 °C. Measured unbound fractions of PHT increased after 2 weeks of storage at 5 °C or –20 °C by 24.1% and 26.6%, respectively. Total concentrations of VPA decreased after 2 weeks of storage at 5 °C or –20 °C by 3.3% and 0.7%,

respectively. Measured unbound fractions of VPA increased after 2 weeks of storage at 5 °C or –20 °C by 41.2% and 31.7%, respectively. After adjusting the pH with PBS, all measured unbound fractions increased over twofold, while there was no difference in total concentrations.

Influence of storage time

The unbound fraction of PHT ($n = 3$) was not affected by 7 days of storage time of the plasma sample before UF ($\chi^2(3) = 1.8$, $p = 0.62$). Conversely, the measured unbound fraction of VPA ($n = 3$) seemed to increase when UF was performed at 96 h (27.4%) or 168 h (24.2%), compared to UF immediately after sample collection ($\chi^2(3) = 7.4$, $p = 0.06$). Results are shown in Fig. 3.

Discussion

In this study, the influence of UF temperature, pH and storage time on unbound fractions of PHT and VPA was analysed.

In our single laboratory experiment, higher UF temperatures resulted in an increase of measured unbound fractions for both PHT and VPA. In the national EQC database, this trend was only observed for PHT, which appears to be more affected by UF temperature than VPA. The effect of UF

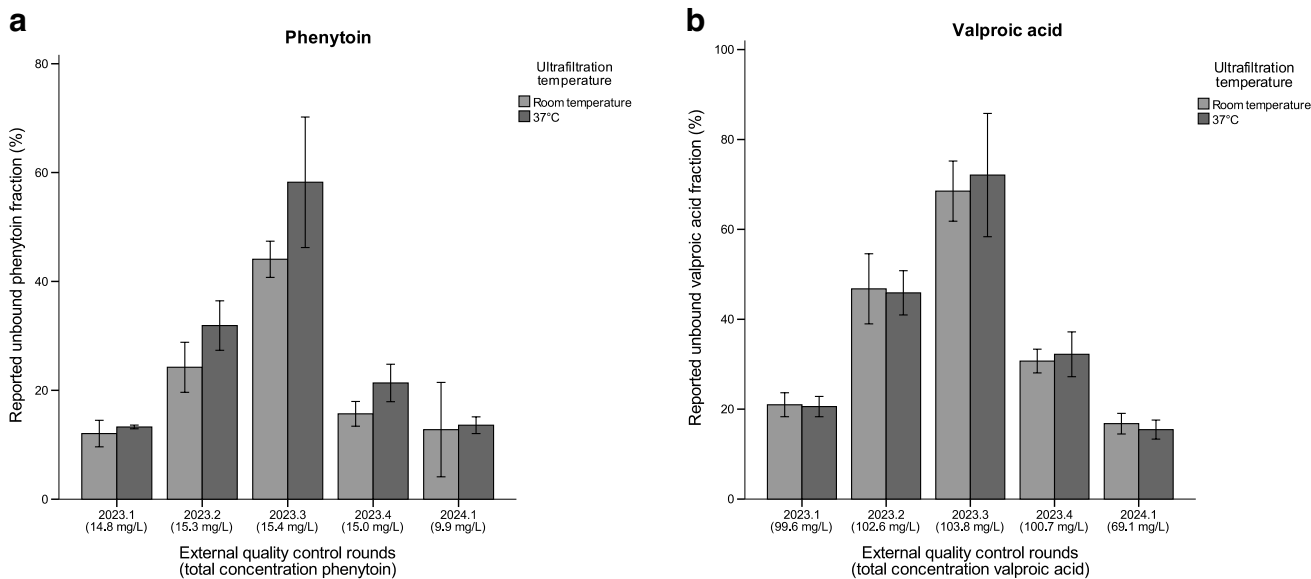
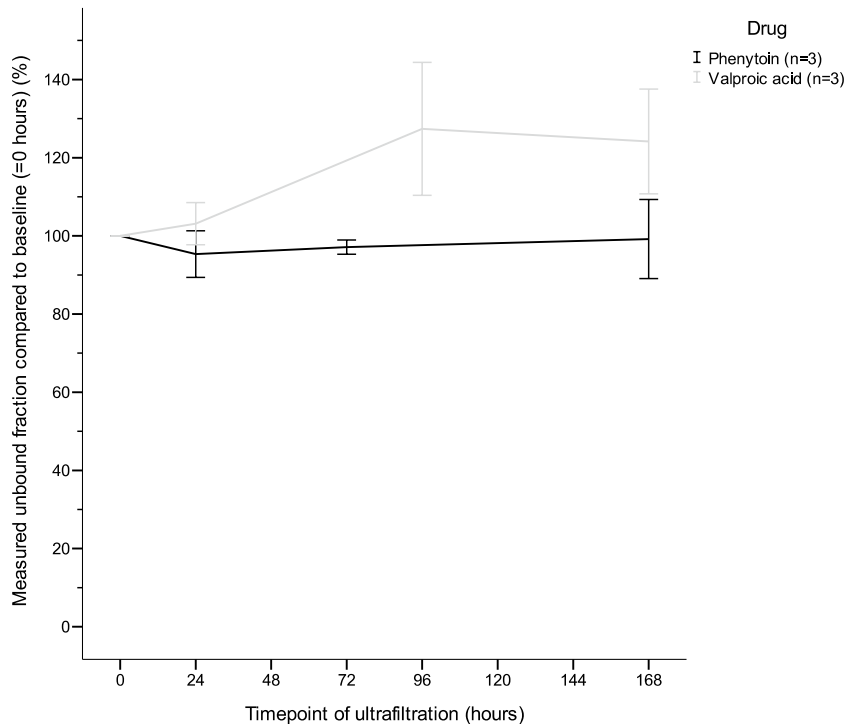


Fig. 2 a Reported mean unbound fractions of phenytoin measured by different laboratories, performing ultrafiltration at room temperature (n=20 for 2023.1, n=19 for 2023.2, n=18 for 2023.3, n=21 for 2023.4 and 2024.1) or 37°C (n=2 for 2023.1, n=4 for 2023.2, n=3 for 2023.3, 2023.4 and 2024.1) on spiked samples from a national external quality control programme. Results are shown as mean ±

standard deviation. **b** Reported mean unbound fractions of valproic acid measured by different laboratories, performing ultrafiltration at room temperature (n=20 for 2023.1, n=22 for 2023.2, n=19 for 2023.3, n=23 for 2023.4 and 2024.1) or 37°C (n=3 for all rounds) on spiked samples from a national external quality control programme. Results are shown as mean ± standard deviation

Fig. 3 Measured mean unbound fractions of phenytoin (PHT) and valproic acid (VPA) compared to baseline (=0 h) after conducting ultrafiltration at four different timepoints following sample collection (t=0 h, t=24 h, t=72 (PHT) or 96 (VPA) hours and t=168 h). Results are shown as mean ± standard deviation



temperature on the measured unbound fractions of PHT has been studied before, obtaining results similar to ours. It is found that the unbound fraction increases from 10 to 15% when measured at an UF-temperature of 37 °C compared

to 25 °C [13]. Another study found an increase from 8.3 to 13.0% in unbound fraction when measured at 37 °C compared to 25 °C [14]. To our knowledge, no such research has been conducted for VPA.

Adjusting sample pH to physiological values using PBS proved to be unfeasible for the analysis of unbound fractions of PHT or VPA, as demonstrated in this study. Protein binding is often strongly dependent on pH variations, possibly due to conformational changes of albumin in the pH 6–9 range [10, 15]. For PHT and VPA, both of which are acidic drugs, literature has shown that measured unbound fractions decrease and increase, respectively, with increasing pH [15]. Consequently, it is recommended to measure unbound fractions at a pH of 7.4 (physiological pH). The most straightforward method to achieve this is by using a CO₂ incubator [10]. However, this device is not commonly available in most clinical laboratories. Therefore, we attempted to adjust the pH to physiological levels using PBS. This approach, however, led to an unexpected > twofold increase in measured unbound fractions. Furthermore, using PBS in a ratio of 1:5 was insufficient to reduce pH to 7.4. If more PBS had been used, many of the samples would fall below the lower limit of quantification. Dilution with PBS might influence the apparent association constant for the PHT/VPA-albumin interaction, possibly explaining the observed increase in measured unbound fractions [10]. Future studies could investigate this further through systematic dilution experiments. These results and limitations render the use of PBS ineffective for adjusting the pH to physiological conditions.

In our experiment, the measured unbound fraction of VPA seemed to increase when conducting UF after 4 days of storage at 5 °C. The occurrence of lipolysis in addition to a pH increase, which both increase the unbound fraction of VPA, might be an explanation for these results [12, 15]. Additionally, unknown competitive binding interactions from other substances may have contributed. In contrast, the unbound fraction of PHT remained stable after 7 days of plasma sample storage. Theoretically, the effects of lipolysis (increase of the unbound fraction) and pH increase (decrease of the unbound fraction) on measured unbound PHT fractions may counterbalance each other, resulting in a net-zero effect [12, 15]. However, a small sample size ($n=3$ per drug) was used, and thus, only a trend towards statistical significance was found for VPA. Further research with a larger sample size is necessary to validate these findings and to evaluate their implications for clinical practice.

When investigating the impact of physiological conditions on measured unbound fractions, it is important to consider the conditions on which the reference ranges of PHT and VPA are based.

The internationally accepted therapeutic reference range for total PHT concentration is 10–20 mg/L [1]. The therapeutic range for unbound PHT concentration is 1–2 mg/L, resulting from 90% protein binding at population level, although protein binding has been found to vary between 75 and 90%. Few studies report UF temperature, but those that do make it likely that the 90% protein binding at population level has been assessed at an UF temperature of 25 °C [6, 13, 16].

The therapeutic reference range for total VPA concentration for epilepsy is 50–100 mg/L [1]. The unbound VPA concentration range proposed by the Dutch National Guidelines is 4–15 mg/L [17]. This is based on the unbound fraction of VPA at different total concentration levels, reflecting its saturable protein binding: the unbound fraction increases from 7% at 50 mg/L to 9% at 75 mg/L, 15% at 100 mg/L, 22% at 125 mg/L and 30% at 150 mg/L [2]. It is unclear at which UF temperature or pH these measurements were performed. Given that UF temperature and pH were not reported, it is unlikely that they were adjusted. Although several studies have looked into the value of measuring unbound VPA concentrations on clinical outcomes, to our knowledge, only one study reports UF conditions [18–20]. Wallenburg et al. evaluated the unbound VPA concentration in relation to toxicity while employing UF at room temperature. In this study, it was found that 77.6% of the patients with elevated unbound VPA concentrations, defined as an unbound VPA concentration > 12 mg/L, showed signs of clinical toxicity [20]. To conclude, reference ranges for both PHT and VPA appear to be based on measurements that were not adjusted for physiological conditions.

Our study has several limitations. Firstly, the use of PBS for pH adjustment was not feasible, preventing any definitive conclusions regarding the influence of sample pH on measured unbound fractions of PHT and VPA. Additionally, the small sample size in our experiment investigating the influence of storage time necessitates further research with a larger sample size to validate our findings. For future studies, it would also be interesting to investigate additional factors that are not directly related to physiological conditions but could nonetheless affect ultrafiltration, such as centrifugation duration, centrifugal force and filter pre-washing [21].

Conclusion

This research highlights the importance of standardised UF conditions to ensure uniform measurement of unbound fractions of PHT and VPA. Performing UF at a temperature of 37 °C more accurately reflects physiological conditions. Our research demonstrates that UF at 37 °C, as opposed to room temperature, increases measured unbound fractions. This effect was more pronounced for PHT than for VPA. Since current therapeutic ranges seem to be based on data obtained with UF conditions at room temperature, the target therapeutic range when performing UF at 37 °C is currently unknown. Additionally, our research demonstrates that storage time of plasma samples might influence the unbound fraction of VPA. Further clinical research is necessary to establish the optimal unbound concentration ranges for PHT and VPA under physiological UF conditions. It is crucial that such research clearly defines UF parameters such as UF temperature to establish reliable reference ranges and

considers the potential effects of sample storage conditions such as lipolysis and pH changes on unbound drug fractions.

Author contributions P.P.T. designed the study, analysed and interpreted the data and wrote the manuscript. M.v.L., M.J.D., N.G.L.J and M.M. designed the study, interpreted the data and reviewed the manuscript. R.M. supplied EQC data and reviewed the manuscript. All authors read and approved the final manuscript.

Data availability No datasets were generated or analysed during the current study.

Declarations

Ethics approval Patient samples collected for routine TDM of PHT or VPA were used of patients that provided general consent for the use of their data for research purposes upon admission to the hospital. Patient samples and data were processed anonymously.

Competing interests The authors declare no competing interests

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