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Research paper

Temperature effect on serum protein binding kinetics of phenytoin in monotherapy patients with epilepsy

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Abstract

The effects of temperature on the binding kinetics of phenytoin (PHT) to serum proteins were determined in patients with epilepsy. Serum samples examined in the study were obtained from 59 patients (31 male, 28 female) with epilepsy on PHT monotherapy. Their age ranged from 3 to 64 years (mean (SD), 23.3 (16.3) years). Protein binding of PHT was evaluated by ultrafiltration under current routine laboratory conditions (25 ± 3°C) or at a temperature of 37°C. The in vivo binding parameters of PHT to serum proteins were determined using a binding equation derived from the Scatchard equation for a one-site binding model. Significant differences were observed in serum concentrations of unbound PHT between paired data ($P < 0.05$). The mean association constant (K) of PHT to serum proteins is 0.011 μM^{-1} at 25 ± 3°C and 0.006 μM^{-1} at 37°C, while mean total concentration of binding sites ($n(Pt)$) is 1002 μM for 25 ± 3°C and 1112 μM for 37°C. Significant differences were observed in the binding kinetics of PHT to serum proteins for the different temperature conditions of ultrafiltration ($P < 0.05$). Our study confirms that binding affinity for PHT-serum protein interaction is approximately 45% lower at 37°C than at 25 ± 3°C and consequently, binding potential ($K \bullet n(Pt)$) is approximately 39% lower at 37°C than at 25 ± 3°C.

Keywords: Phenytoin; Temperature effect; Protein binding kinetics; Epilepsy

1. Introduction

Phenytoin (PHT) is effective for the treatment of primary or secondary generalized tonic-clonic epilepsy, and elementary-partial and complex-partial seizures as well as status epileptics [1]. More than 90% of PHT binds to plasma proteins, mainly albumin [2,3] and exhibits a relatively small inter-subject variation in the unbound plasma fraction, near or within the usual therapeutic concentration ranges [4,5]. Unbound plasma PHT concentration reflects the level in the cerebrospinal fluid [6,7]. Therefore, the determination of binding kinetics in PHT-plasma protein interaction is useful in clinical practice, because the correlation with clinical toxicity is much stronger when unbound rather than total PHT concentrations are considered [8].

The separation of protein bound from unbound antiepileptic drugs in serum or plasma is usually carried out by the ultrafiltration technique in the routine situation. It has been shown that PHT binding to albumin decreases with temperature [9,10]. However, the effects of temperature on binding kinetics of PHT to albumin are not fully determined in patients with epilepsy.

In vitro studies show that PHT has a single population of binding sites on the albumin molecule [11]. Therefore, the

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relationship between total and unbound serum PHT concentrations can be expressed by using a binding equation that was derived from the Scatchard equation, as was the case in the relation between total and unbound serum concentrations of valproic acid [12,13]. Serum albumin concentrations in children reach adult levels in the first year of life [14]. Thus, it is predicted that in children above 1 year of age, the binding kinetics of PHT to serum albumin may be similar to those of adult subjects.

Using this binding equation in the present study, we determined the in vivo population binding parameters of PHT to serum proteins under two different conditions of temperature for ultrafiltration in patients with epilepsy receiving monotherapy.

2. Subjects and methods

2.1. Subjects

Serum samples examined in the study were obtained from 59 patients (Table 1). The patient's ages ranged from 3 to 64 years (3–15 years, 31 subjects; 16–44 years, 18 subjects; 45–64 years, 10 subjects). All were receiving only PHT as their treatment for epilepsy. Biochemical screenings showed their renal and hepatic functions as normal.

Steady state condition for PHT had been already attained, and all patients had taken the same dose of PHT for more than 4 weeks prior to the study. Patients did not take any other chronic medications. Blood samples from each patient were obtained at approximately 3 h after the morning dose of PHT with their usual breakfast. These samples were allowed to clot, and then the serum was separated. All samples were obtained as part of a routine therapeutic monitoring approved by the local ethical committee.

2.2. Sample analysis

Serum levels of total and unbound PHT were determined by a fluorescence polarization immunoassay (TDx; Abbott Laboratories, Chicago, IL). The day-to-day coefficient of variation (CV) of the total PHT assay was 3.3% at 29.7 μM, 3.0% at 59.5 μM, and 2.1% at 118.9 μM. The CV of the unbound PHT assay was 3.0% at 5.9 μM, 3.8% at 9.9 μM, and 1.3% at 13.9 μM.

2.3. Protein binding study

Protein binding of PHT was evaluated by ultrafiltration with a commercially available MPS-3 device (Amicon, Tokyo, Japan) [15] under current routine laboratory conditions (25 ± 3°C) or at a temperature of 37°C. All serum samples were ultrafiltered as soon as possible after separation from blood, because pH changes affect the serum protein binding of drugs [16]. The degree of protein binding was calculated as the ratio of drug in the ultrafiltrate to that in serum and was expressed as the unbound fraction.

2.4. Estimation of binding parameters

The in vivo binding parameters of PHT were determined in all patients by the naive-pooled data method. Data analysis were performed using the SYSTAT statistical package [17], as was the case in the studies of Anderson et al. [18] or Scheyer et al. [19] for the determination of valproic acid binding parameters to plasma proteins. The association constant (K) and total concentration of binding sites ($n(Pt)$) were estimated by iteratively reweighed least squares regression analysis to the binding equation shown below (Eq. (1)) [12,13] derived from the Scatchard equation for a one-site binding model.

$$Cf = \frac{1}{2}\{Ct - n(Pt) - 1/K + [(n(Pt) - Ct + 1/K)^2 + 4Ct/K]^{\frac{1}{2}}\} \quad (1)$$

where Ct is the concentration of total PHT in serum, and Cf is the concentration of PHT not bound to proteins. For estimates of binding parameters of PHT to serum proteins, we used the data reported by Monks et al. [11] as the initial values. Binding potential ($K \bullet n(Pt)$) was also determined.

2.5. Calculation of theoretical minimal unbound serum fraction

The unbound serum fraction (fu) of drugs with a single population of binding sites on serum proteins depends on the association constant for the drug-protein interaction, and the concentration of free proteins (P) according to Eq. (2) [20]:

$$fu = \frac{1}{1 + K \cdot P} \quad (2)$$

and

$$P = n(Pt) - Cb \quad (3)$$

$$Cb = Ct - Cf \quad (4)$$

Table 1
Demographic data and serum PHT concentration of the patients

	Mean ± SD	Range
No. of patients	59	
Sex (m/f)	31/28	
Age (years)	23.3 ± 16.3	3–64
Serum PHT concentration (μM)[a]		
Total	44.8 ± 28.9	4.2–111.8
Unbound		
at 25 ± 3°C	4.0 ± 2.6	0.2–10.7
at 37°C	6.1 ± 4.0	0.4–15.5

[a]The mass [(μg/ml)-to-molar (μM)] conversion factor for serum PHT concentrations was 3.964.

where *Cb* is the concentration of PHT bound to serum proteins. Thus, by substitution Eq. (3) to Eq. (2):

$$fu = \frac{1}{1 + K(n(Pt) - Cb)} \quad (5)$$

The theoretical minimal unbound serum fraction of PHT can be defined as a value of *fu* at 0 μM of bound serum PHT concentration and therefore, is calculated by the following Eq. (6):

$$fu = \frac{1}{1 + K \cdot n(Pt)} \quad (6)$$

2.6. Statistical analysis

The Wilcoxon signed-rank test was used for comparison of paired unbound serum PHT concentrations determined in different conditions of ultrafiltration at a temperature of 25 ± 3 or 37°C. Comparison of binding kinetics of PHT to serum proteins was made by *F*-test, which was an approximation to the likelihood ratio test, to determine differences between data examined at different conditions of ultrafiltration. The predetermined level for significance was $P < 0.05$.

3. Results

The demographic data and serum PHT concentration of the patients are shown in Table 1. The mass [(μg/ml)-to-molar (μM)] conversion factor used for serum PHT concentrations was 3.964 [21].

Serum concentration of albumin was within the normal range (37.0–51.7 g/L) in all patients. The mean serum concentration of albumin was 45.6 g/L (range 37.1–51.2 g/L). Significant differences were observed in serum concentrations of unbound PHT between paired data ($P < 0.05$).

The mean binding parameters characterizing PHT binding kinetics to serum proteins are shown in Table 2. The affinity of PHT to serum proteins is approximately 1.8 times higher at a temperature of 25 ± 3°C than at 37°C, while the total concentration of binding sites seems to be apparently similar between the two temperatures. Significant differences were observed in binding kinetics of PHT to serum proteins for the different temperature conditions of ultrafiltration ($P < 0.05$). Consequently, the theoretical minimal unbound serum fraction of PHT was approximately 1.6 times higher at a temperature of 37°C than at 25 ± 3°C.

Fig. 1 shows the relationships between total and unbound serum PHT concentrations obtained by applying binding parameters determined in different conditions of ultrafiltration at a temperature of 25 ± 3 or 37°C in Table 2 to Eq. (1). The relationship between total and unbound PHT concentrations was more curvilinear at 25 ± 3°C than at 37°C.

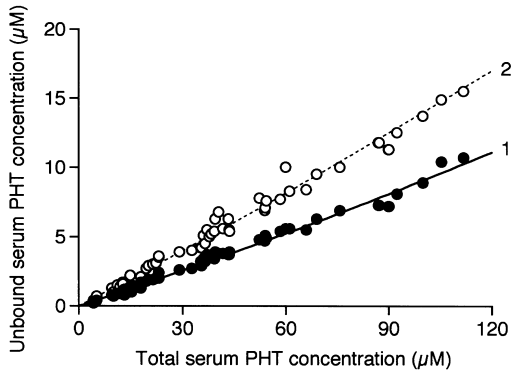

Fig. 1. Relationship between total and unbound PHT concentrations simulated by using the binding parameters determined in different conditions of ultrafiltration at a temperature of 25 ± 3 or 37°C. (1) Binding equation at 25 ± 3°C (●) ($n = 59$), $Cf = (1/2)\{Ct - 1002 - 1/0.011 + [(1002 - Ct + 1/0.011)^2 + 4Ct/0.022]^{1/2}\}$. (2): Binding equation at 37°C (○) ($n = 59$), $Cf = (1/2)\{Ct - 1112 - 1/0.006 + [(1112 - Ct + 1/0.006)^2 + 4Ct/0.006]^{1/2}\}$.

4. Discussion

The effects of temperature on in vivo binding kinetics of PHT to serum proteins were determined in the epileptic patient population with wide age ranges receiving monotherapy. The results show that there are significant differences in binding kinetics of PHT to serum proteins for the data obtained at different temperature conditions of ultrafiltration. Therefore, it is clear that temperature has a significant effect on binding kinetics of PHT to serum proteins.

In an in vitro study of PHT binding to plasma proteins, Lunde et al. [9] found that the unbound plasma fraction of PHT determined by the ultrafiltration method was considerably greater at 37°C than at room temperature (23–26°C). Higher binding under a current routine laboratory condition (i.e. room temperature) may be accompanied with changes in the association constant and/or the total concentration of binding sites. Allison et al. [22] showed significant differences in PHT-serum protein binding affinity as a function of temperature. It appears that the differences between different conditions of temperature at ultrafiltration in binding affinity of PHT to serum proteins are relatively larger than those in binding capacity and therefore, effects of temperature on binding kinetics may be greater in binding affinity than in binding capacity. Our results also demonstrate an approximate 45% reduction in binding affinity of PHT to serum proteins as the temperature increases from 25 ± 3 to 37°C and consequently, binding potential at 37°C is approximately 39% lower compared with that at 25 ± 3°C.

To examine the implication of our study in relation to therapeutic drug monitoring for patients, we calculated theoretical target values for unbound serum PHT concentration using theoretical minimal unbound serum fraction of PHT shown in Table 2. The appropriate therapeutic concentration ranges for total serum PHT are 40–80 μM [23] and therefore, the estimations of target ranges for unbound serum

Table 2

Mean binding parameters and theoretical minimal unbound serum fraction of PHT[a]

Temperature (°C)	Number	K (μM^{-1})	$n(Pt)$ (μM)	$K \cdot n(Pt)$	fu
25 ± 3	59	0.011	1002	11.022	0.083
37	59	0.006	1112	6.672	0.130

[a] K, association constant; $n(Pt)$, total concentration of binding sites; $K\,n(Pt)$, binding potential; fu, theoretical minimal unbound serum fraction.

PHT concentration are 3.3–6.6 μM for 25 ± 3°C and 5.2–10.4 μM for 37°C. Our results show that correction should always be made for temperature effect when applying in vitro data to therapeutic situations in vivo.

Binding potential is a parameter to reflect the capacity of the serum proteins for drug-binding site interaction [24]. As changes in unbound serum fraction of drugs can occurred with changes in binding potential, it is needed to determine which binding parameters of association constant or total concentration of binding sites have significant effects on changes in binding kinetics of drugs to serum proteins. Our study confirms that binding affinity for PHT-serum protein interaction is approximately 45% lower at 37°C than at 25 ± 3°C and consequently, binding potential is approximately 39% lower at 37°C than at 25 ± 3°C. Each laboratory should determine its own therapeutic range for unbound serum PHT under conditions best suited for its particular circumstances, and should consistently report unbound fractions determined at the same temperature.

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