Significant Inhibition of Protein Binding of Phenytoin

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Authors' contributions

This work was carried out in collaboration between all authors. Authors SA and JF performed the study and wrote the protocol. Author HO wrote the first draft of the manuscript. Author MB designed the study and managed the analyses of the study and the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aim: This research was aimed to assess the potential for inhibition of protein binding of phenytoin by tizoxanide in human plasma. Phenytoin was used because studies have proven to be a highly plasma protein-bound drug with a narrow therapeutic index and non-linear pharmacokinetics. Tizoxanide is another highly protein-bound active metabolite of an anti-infective prodrug nitazoxanide. Both drugs are expected to be administered together due to their therapeutic indications.
Study Design: The study was divided into two phases. Phase 1 produced a reference line of phenytoin protein binding values. Phase 2 was conducted to reveal the effect of the interactant tizoxanide on the protein binding of phenytoin. The results obtained from phase 1 were compared with those of phase 2.
Methodology: Protein binding of phenytoin was studied using a centrifugal ultrafiltration method. Protein binding of three phenytoin concentrations was studied: 25, 50, and 100 µg/mL. The concentrations of phenytoin were analyzed by validated HPLC method. Each experiment was performed in triplicate.

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Results: Co-administration of tizoxanide significantly inhibited protein binding of phenytoin for all concentrations tested. Tizoxanide increased unbound fraction (f_u) of phenytoin by 4.4, 3.7, and 2.8-fold for concentration of 25, 50, and 100 µg/ml, respectively. Phenytoin was displaced from protein binding sites what resulted in amplified unbound plasma levels of the antiepileptic drug.
Conclusion: Tizoxanide significantly inhibited protein binding of phenytoin in human plasma. The interaction could potentially result in altered elimination and increased toxicity of phenytoin leading to neuronal side effects and hypersensitivity reactions. Caution also should be taken when administering nitazoxanide concurrently with other highly plasma protein-bound drugs, especially drugs with narrow therapeutic indices, as competition for binding sites may occur.

1. INTRODUCTION

Patients treated with antiepileptic drugs experience variability in therapeutic outcomes including adverse effects. This is due to genetic polymorphisms affecting metabolizing enzymes and drug transporters, plasma/tissue protein binding, and drug-drug interactions [1]. In reference to genetic polymorphisms, ultra-rapid, intermediate, and poor metabolizers demonstrate differences in the CYP450 enzymatic process and therefore, show variabilities in therapeutic response and drug toxicity[1]. Plasma protein binding, another determinant of a drug's therapeutic effect, can affect pharmacokinetics (PK) and pharmacodynamics (PD) of drugs [2]. Drugs in the body exist in two forms bound and unbound (free). Bound drugs do not elicit a therapeutic response because bound drugs are not dynamically distributed to target organs and don't have affinity to target receptors. However, free drugs reach their active sites, interact with receptors and produce therapeutic effect. Therefore, only unbound drugs are predictors of a clinical efficacy or toxicity.

Plasma proteins include albumins, α, β, and γ globulins, lipoproteins, and glycoproteins [3]. The concentration of albumins is much greater than concentrations of other proteins in human plasma. Binding to blood proteins depends on the physicochemical properties of drugs. Acidic compounds are typically bound to albumins, while basic medications connect to globulins.

Plasma protein binding interactions are categorized as displacement reactions. Displacement interactions can be specific or non-specific, competitive or non-competitive. Competitive displacements are leading drug-drug reactions among all protein binding interactions. The consequence of the drug-drug reaction is increased free plasma concentrations of the displaced medication. In turn, this impacts drug's pharmacokinetics parameters: volume of distribution and clearance [3]. Outcome of the protein binding interaction can be dangerous if the displaced medication is highly bound to blood proteins. Additional clinical risks include non-linear pharmacokinetics and/or narrow therapeutic window of the affected drug. Such drug-drug interaction may lead to exaggerated therapeutic response as well as to acute and chronic toxicity. For this reason, each drug interaction is unique and should be analyzed on a case-by-case basis. The simplified approach to protein binding interactions is not ideal as each active pharmaceutical ingredient possess pharmacologically independent characteristics. Measurement rather than estimation of free drug levels should be used to achieve safety and efficacy of highly protein bound medications.

Phenytoin (PHT), 5,5-diphenylimidazolidine-2,4-dione (Dilantin™) is an anticonvulsant medication indicated for the management of generalized tonic clonic seizures, complex partial seizures, and for the prevention of seizures after head trauma and neurosurgery [4]. Multiple unlabeled indications of phenytoin include the management of trigeminal neuralgia, syndrome of inappropriate antidiuretic hormone (SIADH), torsade de pointes, and arrhythmias [4]. The drug is rapidly distributed to the brain where an equilibrium in plasma and brain concentrations is achieved. Phenytoin is metabolized by CYP2C9 and CYP2 C19 enzymes and is a broad-based inducer of cytochrome P450 system. Phenytoin is susceptible to metabolic drug interactions. Cimetidine, valproic acid, amiodarone, chloramphenicol, isoniazid, disulfiram, and omeprazole inhibit phenytoin metabolism and increase phenytoin blood concentrations [5]. As inducer PHT increases metabolism of some narrow therapeutic ranges drugs such as phenobarbital, cyclosporine, carbamazepine, tacrolimus, and warfarin [5]. Moreover, phenytoin is narrow therapeutic range drug with nonlinear, Michaelis-Menten kinetics [4,6,7]. The clearance and half-life of phenytoin are not constant and

depend on phenytoin plasma levels. Studies discovered that phenytoin is approximately 90% bound to plasma albumins while the unbound form is responsible for pharmacologic activity [7,8,9,10]. Since phenytoin is highly bound to albumins, conditions that affect albumin levels can also affect the unbound phenytoin concentrations. Disease-causing hypoalbuminemia such as end-stage renal disease (ESRD), burns, hepatic cirrhosis, nephrotic syndrome, pregnancy, and cystic fibrosis increase the fraction of unbound phenytoin [6,11]. High free levels of PHT can lead to exaggerated pharmacologic response and toxicity. Endogenous (bilirubin) and/or exogenous compounds might alter protein binding of phenytoin as well. Drugs that are highly bound to albumins and cause inhibition of protein binding of PHT include warfarin, valproic acid, and some highly bound nonsteroidal anti-inflammatory medications [12,13,14]. Unbound phenytoin plasma concentrations should be measured in patients with altered phenytoin plasma protein binding [15]. When unbound phenytoin concentrations are unavailable, corrected phenytoin levels typically are calculated.

Nitazoxanide, also known by the brand names Alinia® and Annita®, is a synthetic nitrothiazolyl-salicylamide derivative belonging to a class of thiazolides [16,17]. Nitazoxanide is a broad-spectrum antiparasitic and antiviral drug that is used for the treatment of various helminthic, protozoal, and viral infections [16,18,19,20,21]. *Recently* nitazoxanide was purposed for the treatment of influenza [20,22] and also was investigated as a potential therapy for chronic hepatitis B and C, rotavirus and norovirus gastroenteritis [20,23,24,25,26].

Nitazoxanide is a prodrug that rapidly hydrolyzed to an active metabolite tizoxanide (TZX) [17,27]. The half-life of nitazoxanide in the plasma is very short, approximately 6 minutes [28]. Tizoxanide is highly bound to plasma proteins (>99%), mostly to plasma albumin, as result of its acidic property [29,30]. The half-life of TZX in plasma is approximately 1.5 h [31,32].

Nitazoxanide is a compound that is expected to be co-administered with phenytoin clinically and its active metabolite has the potential to alter phenytoin protein binding. No drug-drug protein binding interaction studies have been reported for phenytoin and nitazoxanide and/or tizoxanide

The aim of this research was to identify the possibility of displacement interaction between phenytoin and tizoxanide.

2. MATERIALS AND METHODS

2.1 Materials

Phenytoin, DMSO, methanol, HPLC water, potassium dihydrogen phosphate, and other chemicals were obtained from Sigma-Aldrich (St Louis, MO). Tizoxanide was purchased from Cayman chemicals (Tallinn, Estonia). Human plasma was obtained from Valley Biochemical, Winchester, MA. HPLC filters (0.45 um, 47 mm) were purchased from Pall Life Science (Port Washington, NY). Amicon Centrifree YM-30 centrifugal filter units were obtained from Millipore (Billerica, MA)

2.2 Study Groups

The study was divided into phases 1 and 2. Phase 1 produced a reference line of phenytoin protein binding values. Phase 2 was conducted to reveal the effect of the interactant tizoxanide on the protein binding of phenytoin. The interactant was chosen based on the possibility of a co-administration with phenytoin in clinical practice and its protein binding characteristics. The results obtained from phase 1 were compared with those of phase 2. Each experiment was performed in triplicate.

Analysis of variance (ANOVA) was applied to assess differences in mean values of phenytoin binding. This statistical method determined the significant differences in protein binding between the two study groups. Statistical significance was detected at a p-value<0.05.

2.3 HPLC Method for Phenytoin

The concentrations of phenytoin were analyzed using validated High Liquid Performance Chromatography (HPLC) technique. Peak separation was achieved by C18 column (250 x 4.6 mm, 5 μm, 100 Å) through isocratic elution. The stationary phase was temperature controlled at 25°C. An ultraviolet detection wavelength of 250 nm was used to measure phenytoin peaks. Mobile phase was prepared from 40% phosphate buffer (pH 2.8) and 60% methanol. Other HPLC variables include a constant flow rate of 0.7 mL/min, a run time of 10 minutes, and an

injection volume of 20 µL. Phenytoin retention time was 7.9 minutes

2.4 Protein Binding Determination

A preliminary work was completed to assess nonspecific binding of phenytoin to the micropartition system (Amicon Centrifree YM-30 System). Three concentrations of phenytoin were studied (25, 50 and 100 µg/mL). Ultrafiltration units with aqueous solutions of phenytoin were centrifuged at a relative force of 2000 g for 20 minutes. The ultrafiltrates were used to evaluate nonspecific binding of the drug to the micropartition devices. The concentrations of phenytoin in filtrates were measured by HPLC. Each experiment was done in triplicate.

Following the assessment of nonspecific binding was an analysis of phenytoin binding to plasma proteins. The investigation was performed at physiologic temperature (37°C) and pH. Human plasma was spiked with stock solution of phenytoin to produce samples of the following concentrations: 25 µg/mL, 50 µg/mL, and 100 µg/mL. Tizoxanide was used only for phase 2 trials. Concentration of TZX was 500 µg/mL in each sample. The resulting solutions were incubated at 37°C for 30 minutes. One mL of each sample was added to Amicon Centrifree devices and centrifuged for 20 minutes at 2000 g. The ultrafiltrated samples were analyzed for measuring free phenytoin concentrations by previously mentioned HPLC method. All tests were performed in triplicate.

2.5 Data Analysis

The fraction unbound (f_u) of phenytoin was calculated as a ratio of unbound (free) concentration to total phenytoin concentration in both phase 1 and 2 studies.

$$f_{u = \frac{C_u}{C_t}}$$

Where: f_u is unbound (free) fraction of phenytoin.
C_u is unbound (filtrate) concentration of phenytoin.
C_t is total concentration of phenytoin.

3. RESULTS

Plasma protein binding of phenytoin was evaluated by ultrafiltration technique. Protein binding of three phenytoin concentrations was studied: 25, 50, and 100 µg/mL.

The assessment of nonspecific binding to the micropartition system yielded negligible binding (fraction unbound ~ 1) to the units. Table 1 represents phenytoin protein binding results.

Phase 1 study formed a reference line of phenytoin protein binding values. Mean free fraction value of phenytoin was 0.14 in this phase. This finding was consistent with previously published data [8,9,10]. No significant difference in protein binding was noted within the study group.

Phase 2 was performed to evaluate effect of TZX on protein binding of phenytoin. Mean free fraction value of phenytoin was 0.48 with no significant difference within this study group. Tizoxanide significantly increased unbound phenytoin concentrations by 4.4, 3.7, and 2.8-fold for concentrations of 25 µg/mL, 50 µg/mL, and 100 µg/mL, respectively

4. DISCUSSION

This investigation was performed to evaluate the potential effect of tizoxanide on protein binding of phenytoin. Phenytoin was of particular interest because of its high protein binding, narrow therapeutic window and non-linear pharmacokinetics. Since phenytoin is a highly protein bound drug, it was imperative to analyze the effect of another extremely protein bound drug on unbound concentrations of phenytoin. Tizoxanide fits these criteria and is expected to be co-administered with phenytoin in clinical practice.

The research revealed statistically significant interaction between phenytoin and tizoxanide. TZX significantly inhibited protein binding of PHT in all concentrations tested. Phenytoin was displaced from protein binding sites what resulted in amplified unbound plasma levels of the antiepileptic drug.

The both medications phenytoin and tizoxanide are bound primarily to albumins and competed for the same binding site of the proteins. Albumins have two main drug binding sites, Sudlow site I and Sudlow site II [33,34]. Drug binding to these sites is selective. Tizoxanide primarily binds to Sudlow site I [35]. Since tizoxanide displaced phenytoin, it can be assumed that PHT also binds to site I. The bindings resulted in direct competition for the shared Sudlow site I. This hypothesis requests further examination.

Table 1. Protein binding of phenytoin (PHT) in phase 1 and 2 studies

Study groups	PHT concentrations (μg/mL)	Fraction unbound of PHT*
Phase 1 PHT alone	100	0.17 (0.004)
	50	0.13 (0.001)
	25	0.11 (0.001)
Phase 2 PHT + TZX	100	0.47 (0.005)
	50	0.48 (0.007)
	25	0.48 (0.005)

Data reported as mean (SD)

Non-linear pharmacokinetics makes phenytoin dosing is extremely challenging. Moreover, other drugs may modify the pharmacokinetics of phenytoin by altering its plasma protein binding and/or metabolism. It is commonly recognized that only unbound phenytoin is pharmacologically active. The development of side effects correlates better with free phenytoin concentrations than with total phenytoin concentrations [36,37]. Metabolism of some medications including PHT depends on unbound fractions of the drugs. Protein binding inhibition of drugs with linear PK lead to an increase in free plasma levels, greater extent of metabolism, increased clearance, and lower total concentrations. Such interactions may result in reduced effectiveness of the therapies. However, non-linear PK makes the outcomes of phenytoin protein binding interaction unpredictable. In case of saturated metabolism, the total concentration of phenytoin is often at the same level as before the drug interaction occurred, but unbound phenytoin concentrations are much higher. Such interaction may result in phenytoin poisonousness. If only total phenytoin concentrations are measured, clinicians may be under the impression that no drug interaction happened. Unbound concentrations have to be measured whenever possible with suspected abnormal phenytoin plasma protein binding to avoid phenytoin intoxication.

Previous studies have focused on the interaction between valproic acid and phenytoin. Valproic acid is a highly protein bound anticonvulsant. Phenytoin unbound concentrations were increased by 50% via coadministration with valproic acid [38]. This interaction has produced serious side effects. Data from the present study indicate that tizoxanide increased phenytoin unbound concentration more than 50%. PHT as a narrow therapeutic index drug poses major changes in the pharmacological effect with only small variations in unbound concentrations. Non-linear PK makes the difference even more dangerous and can produce phenytoin

intoxication. Greater uptake of phenytoin into the brain due to increased unbound level can lead to serious neuronal side effects [39]. When highly protein bound drugs are coadministered with phenytoin, complete blood count, liver function, cardiac function, respiratory function and suicidality are among an extensive list of monitoring parameters to be assessed [40].

The protein binding inhibition of phenytoin can lead to changes in unbound concentrations and, as a result, alteration in the clinical effect. To avoid an unexpected change in pharmacologic response, monitoring of unbound concentrations is mandated and dose adjustments should be implemented.

5. CONCLUSION

This investigation provided an important understanding of protein binding displacement interactions. Protein binding drug-drug interaction was detected between phenytoin and tizoxanide. TZX significantly inhibited protein binding of phenytoin in human plasma. Therefore, tizoxanide has the potential to modify protein binding of PHT and rise free phenytoin concentrations in patients. In addition, PHT is a narrow therapeutic range drug and has non-linear pharmacokinetics what make the drug-drug interaction even more complex. The interaction could lead to great variations in therapeutic response and result in amplified toxicity of phenytoin treatment. For these reasons, phenytoin dose adjustment may be necessary when co-administered with nitazoxanide. Further studies are needed to assess clinical significance of the finding as well as find a strategy to improve clinical outcomes and avoid toxicity of phenytoin therapy.

Caution also should be taken when administering nitazoxanide concurrently with other highly plasma protein-bound drugs, especially drugs with narrow therapeutic indices, as competition for binding sites may occur. The pharmacokinetic

alterations may result in complex changes in total and unbound concentrations and drug response.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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