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Studies by biointeraction chromatography of binding by phenytoin metabolites to human serum albumin

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Abstract

Biointeraction studies based on high performance affinity chromatography were used to investigate the binding of human serum albumin (HSA) to two major phenytoin metabolites: 5-(3-hydroxyphenyl)-5-phenylhydantoin (*m*-HPPH) and 5-(4-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH). This was initially examined by conducting self-competition zonal elution experiments in which *m*-HPPH or *p*-HPPH were placed in both the mobile phase and injected sample. It was found that each metabolite had a single major binding site on HSA. Competitive zonal elution experiments using L-tryptophan, warfarin, digitoxin, and *cis*-clomiphene as site-selective probes indicated that *m*-HPPH and *p*-HPPH were interacting with the indole-benzodiazepine site of HSA. The estimated association equilibrium constants for *m*-HPPH and *p*-HPPH at this site were 3.2 ($\pm$1.2) $\times$ 10^3 and 5.7 ($\pm$0.7) $\times$ 10^3 M^{-1}, respectively, at pH 7.4 and 37 °C. Use of these metabolites as competing agents for injections of phenytoin demonstrated that *m*-HPPH and *p*-HPPH had direct competition with this drug at the indole-benzodiazepine site. However, the use of phenytoin as a competing agent indicated that this drug had additional negative allosteric interactions on the binding of these metabolites to HSA. These results agreed with previous studies on the binding of phenytoin to HSA and its effects on the interactions of HSA with site-selective probes for the indole-benzodiazepine site.

Keywords: Phenytoin metabolites; Human serum albumin; High performance affinity chromatography; Biointeraction studies

1. Introduction

Phenytoin is one of the most widely-prescribed drugs for the treatment of epilepsy [1,2]. It has non-linear dose-dependent pharmacokinetics and is mostly excreted in bile as inactive metabolites, which are then reabsorbed from the intestinal tract and excreted in urine [3,4]. The primary metabolites of phenytoin are 5-(3-hydroxyphenyl)-5-phenylhydantoin (*m*-HPPH) and 5-(4-hydroxyphenyl)-5-phenylhydantoin (*p*-HPPH) (see Fig. 1) [4].

Like other drug metabolites, *m*-HPPH and *p*-HPPH have similar structures to their parent drug, which can cause these to affect the distribution, action, and protein binding by phenytoin [4,5]. Because phenytoin exhibits dose-dependent pharmacokinetics, the apparent half-life of phenytoin changes with its dose and serum concentration. This occurs because phenytoin is hydroxylated in the liver by an enzymatic system that is saturable at high plasma levels of this drug, as occurs at therapeutic concentrations [6]. As a result, small increases in this drug's dose may cause larger-than-expected increases in phenytoin's serum concentration and apparent half-life, along with lower levels for its metabolites [6].

When phenytoin is present in blood, it is highly bound to the carrier protein human serum albumin (HSA) [3,7]. A few reports have indicated that phenytoin interacts at the warfarin-azapropazone site of HSA [8,9], while others have noted competition between phenytoin and digitoxin through the digitoxin site on HSA [10]. Recently Chen et al. have reported that phenytoin interacts at several sites on this protein [7], with association equilibrium constants at the indole-benzodiazepine and digitoxin sites of 1.04 ($\pm$0.05) $\times$ 10^4 and 6.5 ($\pm$0.6) $\times$ 10^3 M^{-1} at pH 7.4 and 37 °C; interactions involving allosteric effects and/or direct binding by phenytoin at the warfarin-azapropazone and tamoxifen sites of HSA were also noted.

Besides phenytoin, HSA is known to bind to a wide range of other drugs and endogenous agents [9,11,12]. Although several studies have examined the binding of phenytoin to HSA, there is no known work that has examined the binding of HSA

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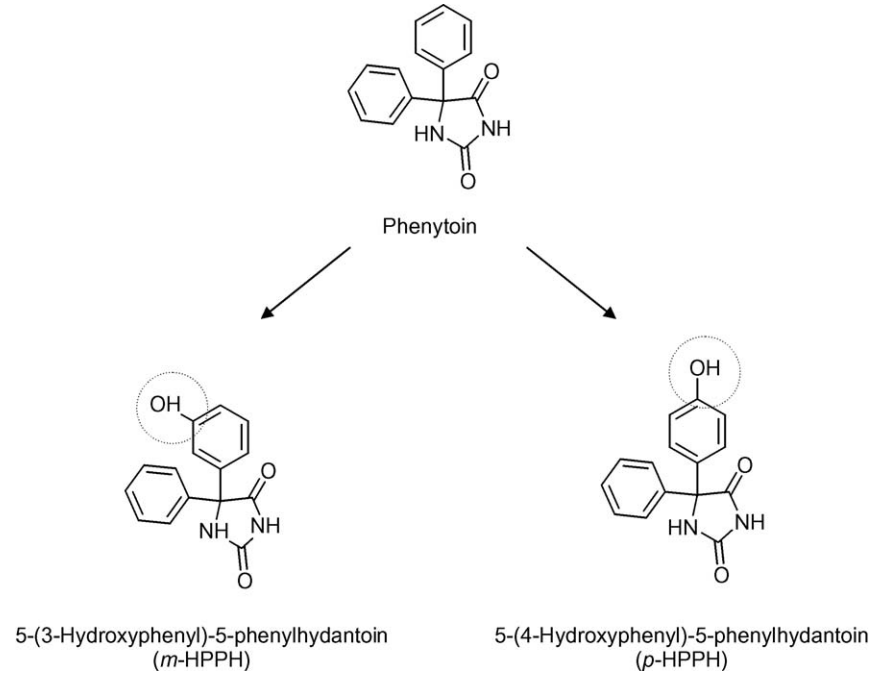

Fig. 1. Structures of phenytoin and its metabolites, *m*-HPPH and *p*-HPPH.

to the phenytoin metabolites *m*-HPPH and *p*-HPPH. This is of interest because 60–80% of phenytoin is metabolized into these forms [13]. The goal of this work is to study the binding of these metabolites to HSA by using high performance affinity chromatography and columns containing immobilized HSA. Numerous reports using HPLC-based HSA columns have reported drug-binding properties that show good agreement with those seen for soluble HSA [7,14,15]; this includes the recent work performed with phenytoin in Ref. [7].

In this current study, self-competition studies with *m*-HPPH and *p*-HPPH will first be used to determine the number of binding regions and association equilibrium constants for these metabolites with HSA. The location of these binding regions will be identified through studies using warfarin, L-tryptophan, digitoxin, and *cis*-clomiphene as site-selective probes [11,16–19]. The competition of these phenytoin metabolites with phenytoin will also be considered. This should result in a more complete picture of the binding of *m*-HPPH and *p*-HPPH to HSA and of the relationship of this binding to that of phenytoin and HSA.

2. Theory

2.1. General model and expressions

The binding of *m*-HPPH and *p*-HPPH to HSA was studied by using the chromatographic method of zonal elution [16,18–22]. In this technique, a competing agent (I) is placed in the mobile phase at a known concentration and continuously applied to an affinity column containing an immobilized ligand (L), such as HSA. Small amounts of an analyte (A) are then injected onto this system and the retention time of the analyte is determined. If I and A compete for a single binding site on L, the following reactions and equilibrium expressions can be used to describe the competition that takes place between these agents in the column.

$$A + L \overset{K_{AL}}{\rightleftharpoons} A - L \tag{1}$$

$$I + L \overset{K_{IL}}{\rightleftharpoons} I - L \tag{2}$$

$$K_{AL} = \frac{\{A - L\}}{[A]\{L\}} \tag{3}$$

$$K_{IL} = \frac{\{I - L\}}{[I]\{L\}} \tag{4}$$

In these equations, K_{AL} is the association equilibrium constant for the binding of A to L, and K_{IL} the association equilibrium constant for the binding of I to L at the site of competition between A and I [16]. The symbol [ ] represents the molar concentration of a solute in the mobile phase, while { } represents a surface concentration.

Eq. (5) has been previously derived for the above system to describe the retention of A as the value of [I] is varied [16].

$$\frac{1}{k} = \frac{K_{IL} V_M [I]}{K_{AL} m_L} + \frac{V_M}{K_{AL} m_L} \tag{5}$$

In this equation, k is the retention factor for the injected solute A, V_M the column void volume, and m_L the moles of binding sites involved in the competition of A with L. If linear elution conditions are present (i.e., the retention factor for A is independent of the amount of injected A), Eq. (5) predicts that a system with direct competition at a single site will give a plot for $1/k$ versus [I] that is linear. From the ratio of the slope to the intercept of this plot it is possible to obtain K_{IL}, the association equilibrium constant for I at its site of competition with A. It is also possible to obtain K_{AL} from the intercept if independent values are available for m_L and V_M [16].