Journal of Chromatography A, 1216 (2009) 3492–3500

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Evaluation of alternatives to warfarin as probes for Sudlow site I of human serum albumin
Characterization by high-performance affinity chromatography

K.S. Joseph, Annette C. Moser, Sara B.G. Basiaga, John E. Schiel, David S. Hage*

Chemistry Department, University of Nebraska, 704 Hamilton Hall, Lincoln, NE 68588-0304, USA

ARTICLE INFO

Article history:
Available online 1 October 2008

Keywords:
Warfarin
Human serum albumin
Sudlow site I
Probe compounds
High-performance affinity chromatography
Drug binding studies

ABSTRACT

Warfarin is often used as a site-specific probe for examining the binding of drugs and other solutes to Sudlow site I of human serum albumin (HSA). However, warfarin has strong binding to HSA and the two chiral forms of warfarin have slightly different binding affinities for this protein. Warfarin also undergoes a slow change in structure when present in common buffers used for binding studies. This report examined the use of four related, achiral compounds (i.e., coumarin, 7-hydroxycoumarin, 7-hydroxy-4-methylcoumarin, and 4-hydroxycoumarin) as possible alternative probes for Sudlow site I in drug binding studies. High-performance affinity chromatography and immobilized HSA columns were used to compare and evaluate the binding properties of these probe candidates. Binding for each of the tested probe candidates to HSA was found to give a good fit to a two-site model. The first group of sites had moderate-to-high affinities for the probe candidates with association equilibrium constants that ranged from 6.4×10^3 M^{-1} (coumarin) to 5.5×10^4 M^{-1} (4-hydroxycoumarin) at pH 7.4 and 37 °C. The second group of weaker, and probably non-specific, binding regions, had association equilibrium constants that ranged from 3.8×10^1 M^{-1} (7-hydroxy-4-methylcoumarin) to 7.3×10^2 M^{-1} (coumarin). Competition experiments based on zonal elution indicated that all of these probe candidates competed with warfarin at their high affinity regions. Warfarin also showed competition with coumarin, 7-hydroxycoumarin and 7-hydroxy-4-methycoumarin for their weak affinity sites but appeared to not bind and/or compete for all of the weak sites of 4-hydroxycoumarin. It was found from this group that 4-hydroxycoumarin was the best alternative to warfarin for examining the interactions of drugs at Sudlow site I on HSA. These results also provided information on how the major structural components of warfarin contribute to the binding of this drug at Sudlow site I.

1. Introduction

The analysis of drug binding to plasma proteins is important in the pharmaceutical industry for characterizing the pharmacokinetics and pharmacological effects of drugs [1–6]. One plasma protein that has been extensively investigated during such work is human serum albumin (HSA) [7]. HSA is the most abundant protein in plasma, with a concentration that ranges from 35 to 50 g/L or 0.6–0.7 mM [1,6–10]. This protein is involved in transporting and distributing many drugs within the body and also binds to a variety of endogenous and exogenous compounds to aid in their transport and to improve their solubility [8–12].

Numerous techniques have been utilized to look at HSA and drug-protein interactions, including ultrafiltration [13], ultracentrifugation [14], equilibrium dialysis [15–17], fluorescence [18,19], UV/vis absorption [19], circular dichroism [20–23], capillary electrophoresis [24–27], surface plasmon resonance [28,29], and nuclear magnetic resonance (NMR) spectroscopy [30,31]. Another technique that has been popular for some time in this type of application is high-performance affinity chromatography (HPAC) [32–36]. HPAC is a specialized form of HPLC that makes use of an immobilized biological ligand (e.g., HSA) as the stationary phase [32–35,37–39]. It has been previously shown that columns containing immobilized HSA are effective models for soluble HSA in drug binding studies, making it possible to rapidly obtain accurate and precise estimates of the association equilibrium constants and number of binding sites for drugs on HSA, while also providing a means for studying drug–drug competition for this protein [32–39]. These properties make HPAC and HSA columns appealing for the high throughput screening of drug binding to HSA.

Both the number of binding sites and affinity of a drug are important in determining the interaction of such an agent with HSA [40].

* Corresponding author. Tel.: +1 402 472 2744; fax: +1 402 472 9402.
E-mail address: dhage@unlserve.unl.edu (D.S. Hage).

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doi:10.1016/j.chroma.2008.09.080

Table 1
Relative cost of warfarin and potential probe candidates for Sudlow site I of HSA.

Analyte	Relative cost (U.S. dollars/g)[a]
R-Warfarin	$72,800
S-Warfarin	$74,800
Racemic Warfarin	$8.38
Coumarin	$0.31
7-Hydroxycoumarin	$1.32
7-Hydroxy-4-methylcoumarin	$0.35
4-Hydroxycoumarin	$0.35

[a] These numbers are based on 2007/2008 list prices from Sigma–Aldrich.

This protein is known to contain two major binding sites for drugs (i.e., Sudlow sites I and II) [41,42], as well as several minor binding sites [43]. One way the binding of a drug at a particular site on HSA can be identified is by determining if this drug has direct competition with a specific probe for that site. Warfarin (i.e., 3-(α-acetonylbenzyl)-4-hydroxycoumarin) is an anti-coagulant drug that is frequently used as a probe for Sudlow site I (also often called the warfarin-azapropazone site of HSA) [44]. Warfarin has a relatively high affinity for HSA and well-characterized interactions with this protein [42]. There are, however, several disadvantages to using warfarin in binding studies. For instance, the strong binding of warfarin to HSA can lead to long retention times for this drug on HPAC columns that contain immobilized HSA [45]. In addition, although the two enantiomers of warfarin have the same binding region but slightly different affinities for HSA [12,44,45], it can be expensive to use these separate enantiomers in binding studies (see Table 1); this has lead to the frequent use of racemic warfarin as a probe in many past investigations of solute interactions with HSA [32,33,37,45]. In addition, recent studies have shown that warfarin undergoes a slow conversion in aqueous solution that can lead to measurable shifts in its binding to HSA over time [44].

The purpose of this study is to examine several compounds that are closely related to warfarin in structure with the goal of determining if these might be used as alternative probes for Sudlow site I in drug-protein binding studies. Ideally, a suitable warfarin replacement for high throughput studies should be specific for Sudlow site I and have few non-specific interactions with HSA or the analysis system. This probe should also have a good long-term stability in aqueous solution and be present in only a single form in solution. Fig. 1 shows the various coumarin compounds that will be examined in this study as possible probes for Sudlow site I. These compounds are all achiral, which avoids the possibility of having any differences in binding by separate chiral forms; this property also makes these compounds more cost-effective to use (as illustrated in Table 1) and easier to obtain than the separate enantiomers of warfarin. In this study, the stability for each of these compounds will be examined by NMR spectroscopy. This will be followed by an evaluation of their binding properties for HSA by using HPAC. From the results it will be possible to compare these compounds and determine which might be suitable replacements for warfarin for use in high throughput screening of drug interactions with HSA. The data obtained in this study should also provide clues as to how the various structural features of warfarin and related coumarin compounds contribute to their binding to Sudlow site I.

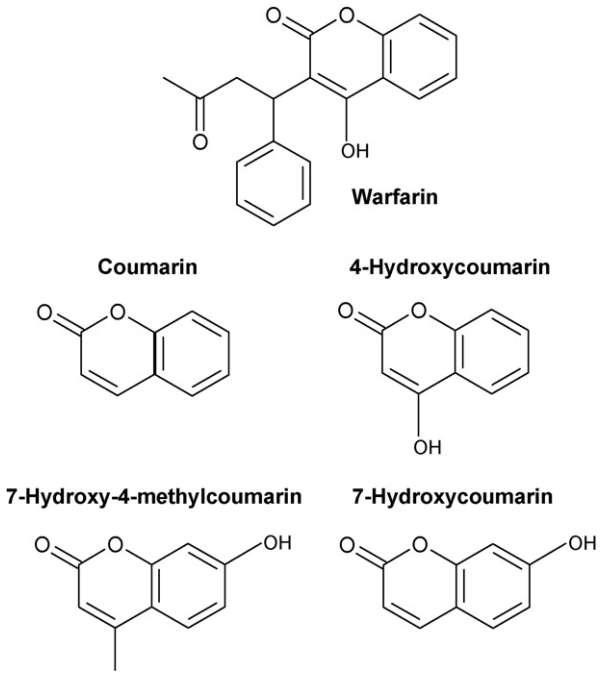

Fig. 1. Structures of warfarin and compounds that were examined as possible alternative probes for Sudlow site I on HSA.

2. Theory

2.1. Frontal analysis

The method of frontal analysis (or frontal affinity chromatography) will be used to determine the number of binding sites and association equilibrium constants for each probe candidate examined in this study. This technique is carried out by continuously applying a solution with a known concentration of the analyte (e.g., a probe candidate) to a column that contains an immobilized ligand (e.g., HSA). As the analyte binds to the ligand, the binding sites in the column become saturated, forming a breakthrough curve like the one shown in Fig. 2(a). If fast association and dissociation kinetics are present, the mean position of this breakthrough curve can be directly related to the concentration of the applied analyte [A], the total moles of active binding sites in the column for the analyte (m_L), and the association equilibrium constant (K_a) for analyte-ligand binding. The following two equivalent equations can be used to relate these terms for a system where the analyte binds to a single type of site on a ligand [32,37].

$$m_{Lapp} = \frac{m_L K_a[\mathrm{A}]}{(1 + K_a[\mathrm{A}])} \quad (1)$$

$$\frac{1}{m_{Lapp}} = \frac{1}{K_a m_L[\mathrm{A}]} + \frac{1}{m_L} \quad (2)$$

In these equations m_{Lapp} is the apparent moles of analyte that are required to reach the mean position of the breakthrough curve at any given concentration of applied analyte, [A]. According to Eq. (2), a plot of $1/m_{Lapp}$ versus 1/[A] for a system with 1:1 binding will make it possible to determine both the binding capacity of the column and the association equilibrium constant by finding the inverse of the intercept and the ratio of the intercept divided by the slope, respectively.

If multi-site binding occurs between the analyte and ligand, a plot prepared according to Eq. (2) will result in a non-linear relationship and produce negative deviations from a linear response at high analyte concentrations (i.e., low values for 1/[A]) [46]. To deal with this situation, Eqs. (1) and (2) can be expanded into the following forms for the case in which an analyte has two different