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Stability of warfarin solutions for drug–protein binding measurements: Spectroscopic and chromatographic studies

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Abstract

Warfarin is commonly used in drug–protein binding studies as a displacement marker for Sudlow site I on the protein human serum albumin (HSA). This study examined the stability of aqueous warfarin solutions prepared for such experiments. This was investigated using NMR spectroscopy and affinity chromatography. It was found by ¹H NMR that warfarin underwent a slow first-order conversion in aqueous solution. The rate of this reaction increased with temperature, giving rate constants at pH 7.4 of 0.0086 h⁻¹ at 25 °C and 0.041 h⁻¹ at 37 °C. It was concluded from further ¹H and ¹³C NMR studies, along with molecular modeling, that this process involved the conversion of the minor cyclic hemiketal form of warfarin to its major cyclic hemiketal. This reaction had a small but measurable effect on the binding of *R*- and *S*-warfarin to HSA, as demonstrated by HPLC using an immobilized HSA affinity column. From this work, general guidelines were developed concerning the usable lifetimes for warfarin that is prepared in aqueous solutions for studies of drug–protein binding.

Keywords: Warfarin, NMR spectroscopy, Affinity chromatography, Stability studies, Conversion kinetics.

1. Introduction

Warfarin is a common oral anticoagulant used in the treatment of venous thrombosis and pulmonary embolism [1–3], as well as in the prevention of prosthetic heart valve thromboembolism [4]. This drug works by inhibiting the production of Vitamin K-dependent clotting factors II, VII, IX, and X [5]. Warfarin is highly bound in circulation, with most of this binding taking place with the protein human serum albumin (HSA) [6]. This interaction occurs at a well-defined region on HSA known as Sudlow site I, or the warfarin-azapropazone site [7, 8]. This binding region is located in subdomain IIA of HSA and binds a large variety of drugs and other compounds [9] with the *R*- and *S*-enantiomers of warfarin having association equilibrium constants at this site of (2.1 and 2.6) × 10⁵ M⁻¹, respectively, at pH 7.4 and 37 °C [10]. Due to its well-characterized binding to HSA, warfarin is often used as a competing agent and probe for the interactions of other drugs and solutes at Sudlow site I on HSA [11, 12].

Although the structure of warfarin is usually drawn as an open chain structure, this is not the only possible configuration for this drug. Warfarin is generally thought to exist in solution as a cyclic hemiketal [13]; this same form has been observed in warfarin crystals [14, 15]. It is also known that in solvents such as dimethylsulfoxide (DMSO) and chloroform an equilibrium exists between three tautomeric structures of warfarin: a major cyclic hemiketal, a minor cyclic hemiketal, and an open chain form (see Figure 1) [16, 17]. In DMSO the reported ratio of these forms is 70:30:0 and in chloroform the ratio is 45:40:15 [16, 17]. In aqueous solution the ratio of the cyclic hemiketal to open chain form is thought to be approximately 20:1 [18], but further identification of the actual structures of warfarin that exist in an aqueous solution has not yet been described.

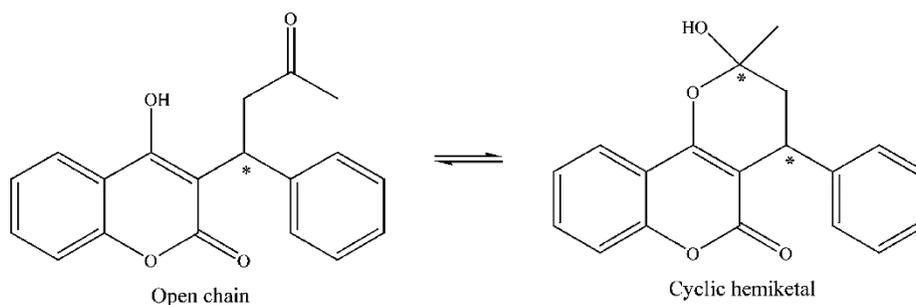


Figure 1. The open chain and cyclic forms of warfarin. A conversion between these forms occurs as a result of an enol-keto tautomerism reaction. The asterisks indicate the location of the chiral centers in these structures. The major and minor hemiketal forms are a result of the formation of an additional chiral center to form two sets of epimers (*R,R*- and *S,S*-warfarin or *S,R*- and *R,S*-warfarin).

The purpose of this study is to examine the stability of warfarin in aqueous solution, to see if there is any long-term change in the form of warfarin in such a solution, and to determine how possible changes in warfarin's structure affect its use in binding studies with HSA. ^1H NMR spectroscopy will initially be used to monitor the stability of warfarin in aqueous solutions under various storage conditions. ^1H NMR, ^{13}C NMR and molecular modeling will then be used to investigate any structural changes in warfarin that are detected. Structurally induced changes in the binding of warfarin to HSA will be examined through the use of an HPLC-based immobilized HSA affinity column. These experiments should provide information concerning the stability of warfarin in aqueous solution and indicate how any changes in warfarin's structure will affect its use in drug-protein binding studies.

2. Experimental

2.1. Reagents

The HSA (Cohn fraction V, 99% globulin and fatty acid free) and racemic warfarin (98% pure) were from Sigma (St. Louis, MO, USA). The Nucleosil Si-300 (7 μm particle diameter, 300 \AA pore size) was obtained from Alltech (Deerfield, IL, USA). All aqueous solutions were prepared using water from a Nanopure water system (Barnstead, Dubuque, IA, USA). The *R*-(+)- and *S*-(-)-enantiomers of warfarin were obtained from Dr. H.H.W. Thijssen of the Univ. Maastricht (Maastricht, The Netherlands). Monobasic potassium phosphate and dibasic potassium phosphate salts were acquired from Mallinckrodt (Hazelwood, MO, USA). Deuterated dimethylsulfoxide (DMSO- d_6) (99.96% pure) was obtained from Aldrich (St. Louis, MO, USA). Deuterium oxide (>98% pure) was purchased from Cambridge Isotopes (Andover, MA, USA).

2.2. Instrumentation

The HPLC system consisted of a Jasco PU-980 pump (Easton, MD, USA) and a SM3100 Milton Roy UV/vis variable wavelength detector (Riviera Beach, FL, USA). Data were collected from this system using Labview 5.1 software (National Instruments, Austin, TX, USA). Injections were performed using a Spectra-system AS3000 autosampler (Riviera Beach, FL, USA) equipped with a 20 μ L sample loop and injection marker. The column temperature was controlled using a Fisher Scientific 9100 refrigerated circulator (Pittsburgh, PA, USA) and an Alltech water jacket. Columns were downward slurry packed at 3000 psi using an HPLC slurry packer from Alltech. The pH meter was an IQ240 pH meter (IQ Scientific Instruments, Carlsbad, CA, USA) equipped with an ion selective field effect transistor (ISFET) probe.

The NMR spectra were acquired on a Bruker DRX Avance 400 MHz NMR using a QNP probe and X-WinNMR as the processing program (Bruker, Billerica, MA, USA). These spectra were analyzed using Nuts from Acorn NMR (Livermore, CA, USA) and XWIN PLOT from Bruker. Sixty-four scans were acquired for ^1H NMR experiments and approximately 2000 scans were acquired for the ^{13}C NMR experiments. The ^{13}C NMR spectra for the cyclic and acyclic forms of warfarin were modeled using ChemDraw 9.0 (CambridgeSoft, Cambridge, MA, USA). The stability of warfarin epimers was examined by using molecular mechanic (MMX) calculations performed with PC Model 7.1 (Serena Software, Bloomington, IN, USA).

2.3. Methods

2.3.1. NMR studies

Deuterated phosphate buffer was prepared by adding 0.0912 g monobasic potassium phosphate to 10.0 mL deuterium oxide to create a 0.067 M phosphate solution. A second 0.067 M phosphate solution was made by adding 0.1167 g dibasic potassium phosphate to 10.0 mL deuterium oxide. These two solutions were then mixed in varying ratios to create solutions with the desired pH (or pD when using the deuterated buffer salts).

A saturated solution (i.e., roughly 200 μM) of racemic warfarin was prepared in the pH 7.4, 0.067 M deuterated phosphate buffer. Immediately following preparation of this solution, a ^1H NMR spectrum was taken of the sample. For the temperature studies performed in this report, a fresh warfarin sample was split into five portions that were then stored for various lengths of time at 5, 12, 25, 37, or 45 $^{\circ}\text{C}$. Two other samples were kept at 25 $^{\circ}\text{C}$ and stored in normal room lighting or in the dark. ^1H NMR spectra for the 5 or 12 $^{\circ}\text{C}$ samples were taken over 1–49 days. For the 25 $^{\circ}\text{C}$ samples, spectra were taken over 1–23 days. Spectra for the 37 or 45 $^{\circ}\text{C}$ samples were acquired over 1–3 days. Warfarin samples used in the pH studies were prepared in a similar manner by placing a saturated solution of warfarin in pH 7.8 or 8.2, 0.067 M potassium phosphate buffer; ^1H NMR spectra of these samples were taken immediately after their preparation, with subsequent spectra being acquired over the following 2 weeks.

In studies examining the mechanism of warfarin conversion, a saturated solution of racemic warfarin (i.e., roughly 500 μM) was placed in a 50:50 mixture of DMSO- d_6 and pH 7.4, 0.067 M deuterated phosphate buffer. Immediately following preparation of this sample, a ^1H NMR spectrum was taken. This solution was then stored at 25 $^{\circ}\text{C}$ and additional spectra were taken over the course of 3 weeks. For ^{13}C NMR studies examining the mechanism of warfarin conversion, a similar sample of racemic warfarin was prepared in a 50:50 mixture of DMSO- d_6 and pH 7.8, 0.067 M deuterated potassium phosphate buffer. A ^{13}C NMR spectrum was acquired for this sample immediately after its preparation; this solution was then stored at 37 $^{\circ}\text{C}$ for 3 weeks, followed by acquisition of a second ^{13}C NMR spectrum.

2.3.2. Chromatography

Diol-bonded silica for preparation of the immobilized HSA column was made from Nucleosil Si-300 silica according to a previous method [19]. HSA was then immobilized onto this support using the Schiff base method, also according to the literature [20]. These procedures result in a support that contains approximately 30 mg HSA/g silica [20] and that has been shown in numerous studies to be a good model of the behavior of HSA in solution [10, 20–26]. The immobilized HSA silica was then packed at 3000 psi into a 10 cm × 2.1 mm i.d. stainless steel column using pH 7.0, 0.1 M potassium phosphate buffer as the packing solvent. Once the column was packed, it was stored at 4 °C in pH 7.4, 0.067 M potassium phosphate buffer. The mobile phase used with this column for drug binding studies was pH 7.4, 0.067 M potassium phosphate buffer applied at 0.5 mL/min. Under these conditions, this type of HSA column is known to have good stability, with less than a 5–10% change in measured association constants being noted over 9 months and/or 500 sample injections [27]. Prior to its use, the mobile phase was filtered through a 0.45 µm cellulose acetate filter and degassed under vacuum for 15 min.

The binding of warfarin to the immobilized HSA column was examined by using zonal elution. This was performed by injecting small amounts of *R*- or *S*-warfarin on the HSA column at known temperatures and determining the retention time of the analyte. This retention, in turn, was used as a measure of the binding strength between warfarin and HSA, as reported in previous studies [10, 20–26]. The elution of warfarin from the HSA column was monitored at 208 nm. The chromatographic studies were performed at 10.0, 25.0, and 37.0(±0.1) °C. The concentration of warfarin in the injected samples was between 2.5 and 10 µM. Under these conditions, no noticeable shifts in peak retention occurred with a change in sample concentration, indicating that linear conditions were present within the column [10, 23]. These studies were performed by using two sets of *R*- and *S*-warfarin samples. One set was injected onto the HSA column immediately after preparation while the other was injected after being heated to 45 °C for 4 days. Retention times were calculated using the first statistical moments of the peaks [23].

3. Results and discussion

3.1. ¹H NMR studies of warfarin stability

The stability of warfarin at pH 7.4 in an aqueous solution was examined using ¹H NMR. A typical spectrum obtained for a fresh solution of this compound is shown in Figure 2. Even though these ¹H NMR results suggested that only one major form was present in a fresh solution of warfarin, it was found that there was a change in this spectrum over time. This is demonstrated in Figure 3 for data collected at 25 °C over 23 days. Similar results were obtained at other temperatures over shorter or longer time scales, as will be discussed later. Although most of the peaks for warfarin remain unchanged over time, it was noted that the doublet originally seen at 3.46 ppm (item “b” in Figure 2) began to slowly disappear over time with a corresponding increase in another doublet at 3.42 ppm.

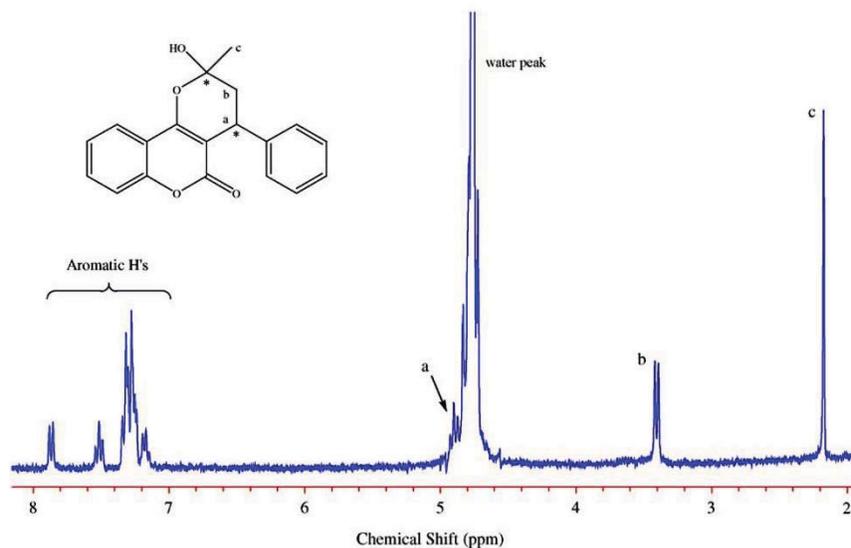


Figure 2. ^1H NMR spectrum for a freshly prepared solution of warfarin in pH 7.4, 0.067 M deuterated phosphate buffer. The asterisks indicate the location of the chiral centers in the cyclic form of this compound. The open chain form would be expected to give a similar splitting pattern (e.g., protons a, b, and c would result in a triplet, a doublet, and a singlet in both the open chain and cyclic forms).

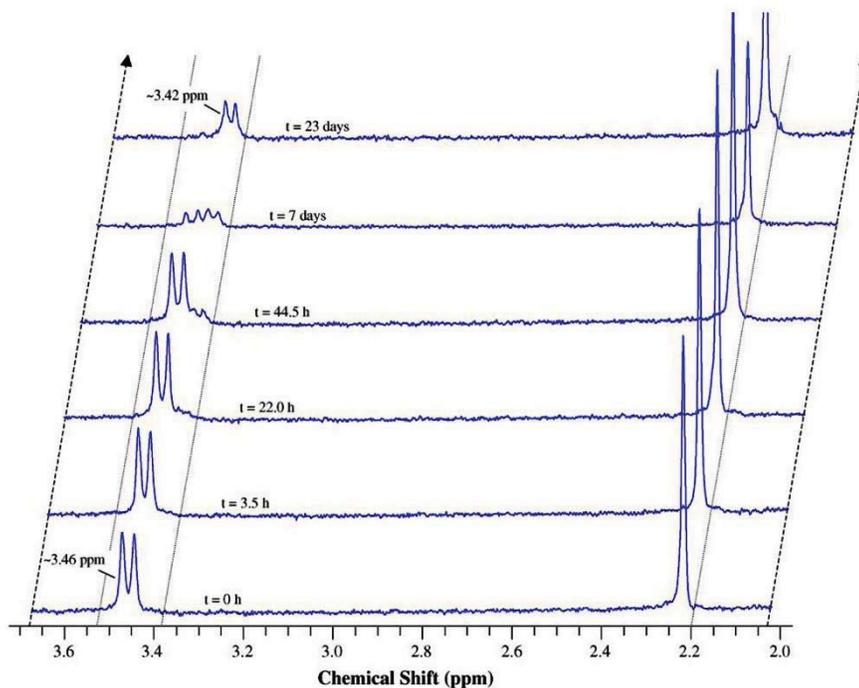


Figure 3. Change in the ^1H NMR spectrum for warfarin over time when stored in pH 7.4, 0.067 M deuterated phosphate buffer at 25 °C. This figure emphasizes the region between 2.1 and 3.7 ppm where a shift in the doublet from 3.46 to 3.42 ppm occurs over time. No other visible changes were noted in other regions of the ^1H NMR spectrum for warfarin under these conditions.

This slight change in the NMR spectrum suggested warfarin was undergoing a change in its structure. Based on these results and previous studies using other solvents, this change could be attributed to several possible processes: (1) base hydrolysis of warfarin; (2) conversion between the cyclic and open chain forms of warfarin; or (3) conversion of warfarin from one cyclic hemiketal to another. Each of these possible reactions will be considered in more detail in Section 3.3.

3.2. Kinetic studies of warfarin stability

The rate at which the change in warfarin structure took place was determined by ^1H NMR. This was accomplished by calculating the peak area ratio of the doublet at 3.46 ppm in Figure 3 versus the peak area of the singlet at 2.1 ppm, which was used as an internal standard since it did not change over time. Based on this ratio of peak areas, plots for zero through second-order kinetic models were constructed to determine the reaction order. The best-fit occurred with a model in which first-order decay was used to describe the disappearance of the doublet at 3.46 ppm. Examples of such plots are shown in Figure 4, where the data were acquired over approximately two to four half lives of the decay process. Correlation coefficients of 0.996–0.999 were obtained over four to five data points for all these plots, giving fits that were significant at greater than the 95% confidence level [27].

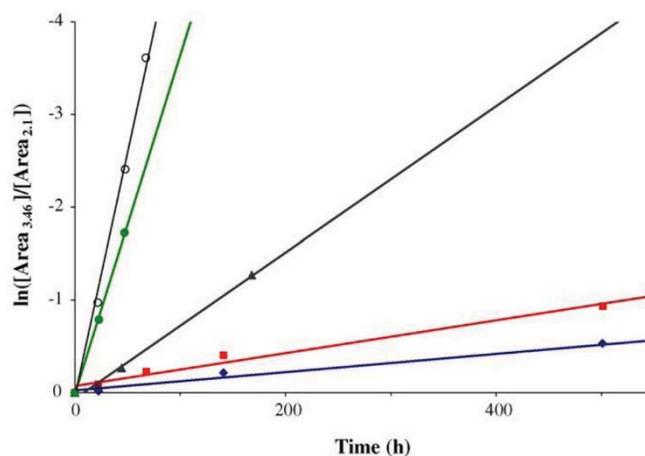


Figure 4. First-order kinetics plots based on ^1H NMR spectra of warfarin obtained at various times for solutions in pH 7.4, 0.067 M deuterated phosphate buffer stored at: 5 °C, (◆); 12 °C, (■); 25 °C, (▲); 37 °C, (○); or 45 °C, (●). The equations for the best-fit lines were as follows: 5 °C data, $y = -0.00099(\pm 0.00004)x - 0.02(\pm 0.02)$ with $r^2 = 0.996$ ($n = 5$); 12 °C data, $y = -0.0028(\pm 0.0002)x - 0.02(\pm 0.01)$ with $r^2 = 0.992$ ($n = 5$); 25 °C data, $y = -0.0079(\pm 0.0003)x + 0.07(\pm 0.03)$ with $r^2 = 0.994$ ($n = 5$); 37 °C data, $y = -0.054(\pm 0.002)x + 0.1(\pm 0.1)$ with $r^2 = 0.996$ ($n = 4$); 45 °C data, $y = -0.037(\pm 0.001)x + 0.02(\pm 0.04)$ with $r^2 = 0.999$ ($n = 3$), where all numbers in parentheses represent a range of 1 standard deviation.

Using the first-order rate constants (k) obtained from Figure 4, an Arrhenius plot was created to examine the temperature dependence of these rate constants. The resulting graph of $\ln(k)$ versus $1/T$ is shown in Figure 5. The correlation coefficient for this plot was 0.970 over five points, giving a fit that was significant at the 95% confidence level. The resulting estimates of k that were determined from Figure 4 and Figure 5 are summarized in Table 1. For instance, the rate constant for conversion of the 3.46 ppm doublet form of warfarin to its 3.42 ppm doublet form at 37 °C was estimated to be between 0.029 and 0.054 h^{-1} (average,

0.041 h⁻¹) from these results. At 25 °C this rate constant was between 0.0079 and 0.0093 h⁻¹ (average, 0.0086 h⁻¹) and at 5 °C it was approximately 0.00099–0.0011 h⁻¹ (average, 0.001 h⁻¹).

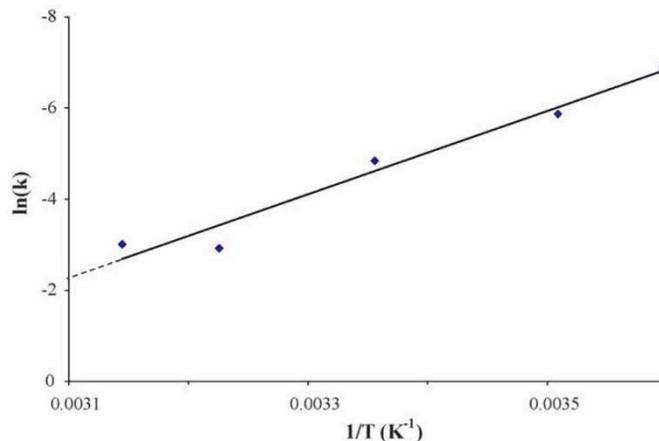


Figure 5. Arrhenius plot showing the temperature dependence of the rate constant k for the conversion of warfarin from a form with a 3.46 doublet to a 3.42 doublet in its ¹H NMR spectrum. The equation for the best-fit line was $y = 8700(\pm 1300)x + 24(\pm 4)$ with $r^2 = 0.970$ over five data points.

Table 1. Rate constants, half-lives and conversion times for warfarin in pH 7.4, 0.067 phosphate buffer^a

Temperature (°C)	Rate constant, k (h ⁻¹)	Half-life, $t_{1/2}$	Time for 5% conversion (h)	Time for 95% conversion (days)
5	0.00099–0.0011	25–29 days	45–52	109–127
12	0.0025–0.0028	10–12 days	18–21	44–51
25	0.0079–0.0093	3.1–3.7 days	5.5–6.5	13–16
37	0.029–0.054	13–24 h	1.0–1.8	2.3–4.4
45	0.037–0.058	12–19 h	0.9–1.4	2.2–3.4

a. The range given for each rate constant reflects the values measured from the first-order kinetic plots in Figure 4 and those estimated from the Arrhenius plot in Figure 5.

Based on the rate constants measured in this study and the temperature dependence of these constants, it was possible to predict the amount of time needed to convert a given fraction of the 3.46 ppm doublet form of warfarin into its 3.42 ppm doublet form. This was used to evaluate the stability of the warfarin by determining the time required for 5% of its initial 3.46 ppm doublet form to be lost, as well as the time needed for 95% of this warfarin to convert into the final 3.42 ppm doublet form. The results are summarized in Table 1. For instance, the time required for 5% of the warfarin to undergo this conversion was an average of 1.4 h at 37 °C, 6.0 h at 25 °C, and 49 h at 5 °C. These values represent the maximum amount of time fresh warfarin solutions can be used before a significant loss of warfarin's original form occurs in pH 7.4, 0.067 M phosphate buffer. Similarly, periods of 3.3, 14.5 and 120 days were needed at 37, 25 and 5 °C to convert 95% of the warfarin to its 3.42 ppm doublet form.

3.3. NMR and molecular modeling studies of warfarin conversion

The next item considered was to determine the nature of the reaction that gave rise to the shift from the 3.46 ppm doublet to the 3.42 ppm doublet in the ^1H NMR spectrum for warfarin. As shown in Figure 6, several reactions were considered as candidates for this conversion process. This included the formation of a warfarin carboxylate through base hydrolysis, the conversion of warfarin between its cyclic and open chain forms by enol-keto tautomerization, and the conversion of warfarin between epimers (i.e., its minor and major cyclic hemiketal forms).

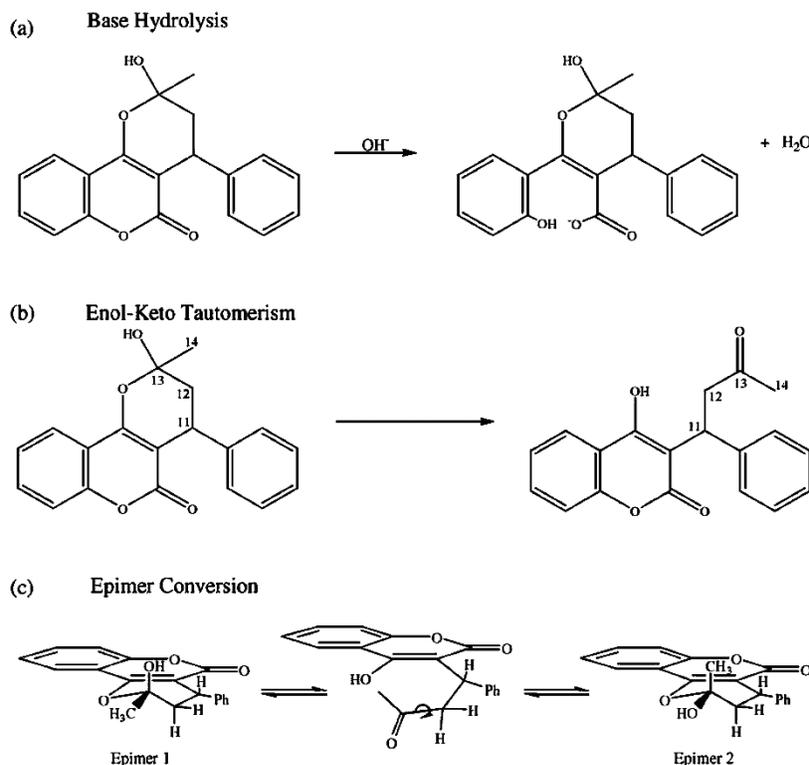


Figure 6. Possible reactions that can give changes in warfarin structure: (a) base hydrolysis of warfarin to form a carboxylate; (b) conversion of the cyclic to open chain forms of warfarin through an enol-keto tautomerism reaction; (c) conversion of warfarin from one epimer to another. In the cyclic form of warfarin, as shown to the left and right of reaction (c), there are two chiral centers are present; this gives the possibility of four stereoisomers through this reaction. In (c), only the epimers for *S*-warfarin are shown; however, an additional pair of epimers would exist in solution containing *R*-warfarin, which would give mirror image structures to the epimers shown for *S*-warfarin. The asterisks designate the presence of chiral centers.

The reactions shown in Figure 6 are written with the cyclic hemiketal being the original form of warfarin in solution (see left of reactions a–c). This is based on data obtained in earlier reports. For instance, it was previously found that the initial ratio of warfarin's cyclic hemiketal and acyclic enol forms in aqueous solution was 20:1 at 25 °C [18]. The cyclic hemiketal form of warfarin has also been observed in other solvents, including dimethylsulfoxide (DMSO) and chloroform [13, 16, 17].

The first possible reaction considered for this conversion was the formation of a warfarin carboxylate through base hydrolysis, as shown in Figure 6a. In this reaction, the ^1H NMR spectra for the original and final forms of warfarin would be quite similar with only a slight shift in peaks "a" and "b" in Figure 2. If peak "a" shifted, this would be difficult to detect due to its close proximity to the water peak in the ^1H NMR spectrum. However, the shift in the peaks at "b" in Figure 2 could correspond to such a reaction. Since the rate of this hydrolysis would depend on the concentration of hydroxide ions, this process would be expected to accelerate in rate by moving to a more basic pH. This was tested by placing warfarin in a series of deuterated phosphate buffers with pH values of 7.4, 7.8 or 8.2, followed by acquisition of ^1H NMR spectra for these samples over several days to monitor the doublet shift from 3.46 to 3.42 ppm. Instead of an increase, it was found that the rate of this reaction decreased from $(7.9(\pm 0.3) \text{ to } 4.86(\pm 0.03)) \times 10^{-3} \text{ h}^{-1}$ at 25°C when going from pH 7.4 to 8.2. Thus, it was determined that base hydrolysis was not a likely candidate for the conversion process being observed in this study. In addition, this information indicated that the acid dissociation of warfarin was also not the cause of this conversion process.

The second possible reaction considered was the conversion of warfarin from a cyclic to open chain form, as shown in Figure 6b. Although warfarin is known to exist in a cyclic form in aqueous solutions, its anticoagulant properties are thought to be due to the open chain form [17]. Furthermore, a crystal structure of warfarin bound to HSA has shown that warfarin is present in an open chain form in this drug-protein complex [28]. The cyclic-to-open chain conversion was examined in this study through the combined use of computer modeling and ^{13}C NMR. Based on the predicted chemical shifts in the ^{13}C NMR spectra, one major difference was noted: the carbonyl carbon (carbon 13 in Figure 6b) in the open chain form had a predicted chemical shift of 208 ppm while the same carbon in the cyclic form had a predicted chemical shift of 92 ppm. These predicted shifts gave good agreement with a previous study of warfarin in DMSO, which gave chemical shifts for carbon 13 in the cyclic form of 99.6–103.4 ppm for the major/minor hemiketals and a chemical shift of 216.5 ppm for the open chain form [13]. Similar studies in chloroform have given chemical shifts for carbon 13 of 99.2 ppm in the major hemiketal (104.3 ppm in the minor hemiketal) and 212.0 ppm in the open chain form [16].

One difficulty associated with acquiring a ^{13}C NMR spectrum for warfarin was the limited solubility of this drug in pH 7.4, 0.067 M potassium phosphate buffer. This meant that even when a ^{13}C NMR spectrum was acquired for such a solution over relatively long times (i.e., overnight), no noticeable peaks were seen for warfarin. DMSO was added to the phosphate buffer to increase warfarin's solubility and ^{13}C NMR signal. The pH of the phosphate buffer was also increased to pH 7.8 to further increase this solubility (also giving a slight decrease in the conversion rate of warfarin from its initial to final forms). ^1H NMR studies were conducted to determine what effects the addition of DMSO had on the warfarin conversion process. When the resulting spectra were compared to those obtained earlier in pH 7.4, 0.067 M phosphate buffer, the same change in doublets was noted but with a decrease in the conversion rate from $(7.9(\pm 0.3) \text{ to } 1.4(\pm 0.1)) \times 10^{-3} \text{ h}^{-1}$ at 25°C . When a ^{13}C NMR spectrum was taken of warfarin in the 50:50 (v/v) mixture of DMSO and pH 7.8, 0.067 M phosphate buffer, a peak was seen at 103.2 ppm in both the original and final forms of warfarin (see Figure 7). Based on the modeling studies, this indicated that warfarin had a cyclic form in both its original and final structures. Thus, it was concluded that the conversion of warfarin between a cyclic and acyclic form was not the process giving rise to the change in warfarin seen in this study.

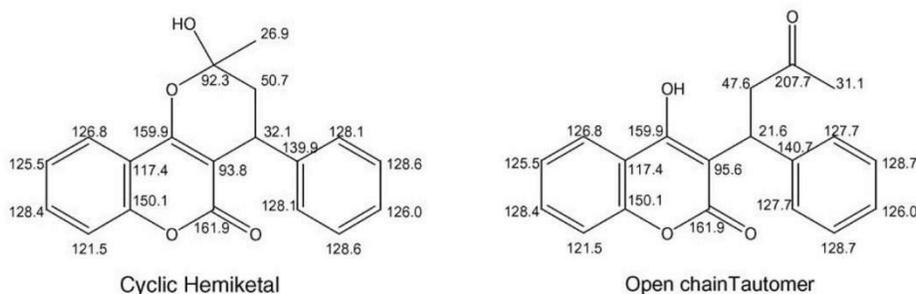
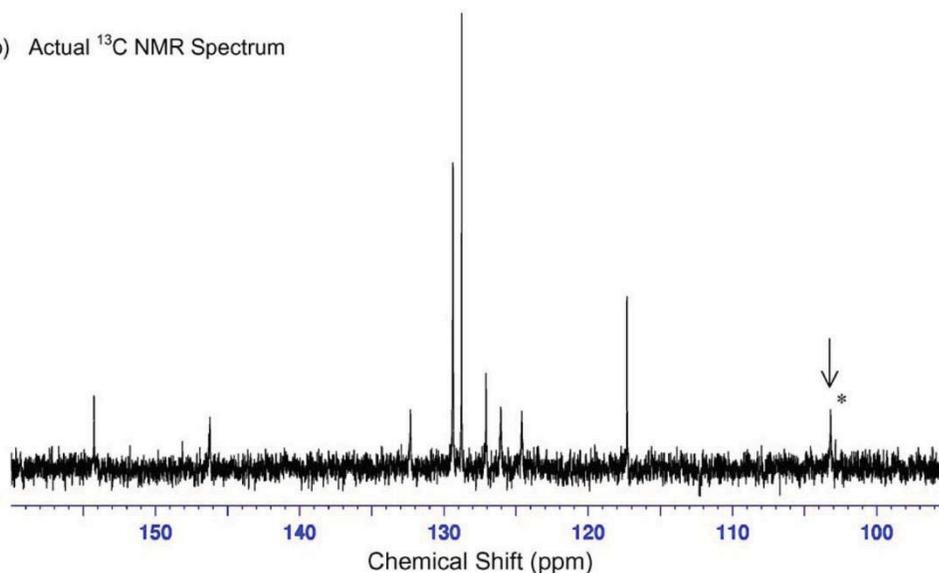
(a) Predicted ^{13}C Chemical Shifts(b) Actual ^{13}C NMR Spectrum

Figure 7. (a) Warfarin structures with estimated ^{13}C NMR chemical shifts from ChemDraw 9.0. (b) ^{13}C NMR spectrum for a freshly prepared solution of warfarin in 50:50 of pH 7.8, 0.067 M deuterated phosphate buffer:DMSO- d_6 . The freshly prepared solution and the converted warfarin solutions gave virtually identical ^{13}C NMR spectra.

The final reaction considered was the conversion between warfarin epimers. This reaction is possible due to the formation of two chiral centers in warfarin when it assumes a cyclic hemiketal form via an enol-keto tautomerism reaction, as shown in Figure 6c. These two chiral centers result in major and minor cyclic hemiketals, or epimers. Mixtures of these warfarin epimers have been noted in other solvents (i.e., DMSO and chloroform), as indicated by two sets of peaks in both ^1H and ^{13}C NMR spectra [13, 16, 17]. Only one set of peaks was seen in this report for the ^{13}C NMR spectrum of warfarin (see Figure 7); this may have been due to a low abundance for one of the epimers and/or the relatively weak signal for warfarin in this spectrum. However, two sets of doublets were noted at 3.46–3.42 ppm in the ^1H NMR spectra for warfarin (Figure 3), as would be expected for a mixture of such epimers.

In previous work with benzylpenicilloic acid, a slow conversion between epimers has been noted in aqueous solution at pH 8.7 [29]. In this case, the conversion from 5*R*,6*R*-benzylpenicilloic acid to 5*S*,6*R*-benzylpenicilloic acid was found to occur over a 5 day period. Although this was not a complete conver-

sion, the ratio of 5*R*,6*R*-benzylpenicilloic acid:5*S*,6*R*-benzylpenicilloic acid did shift from 20:1 to 0.25:1 during this reaction. The approximate rate constant for this conversion was 0.8 h⁻¹. This behavior is quite close to that seen here for warfarin, indicating the epimer conversion is the most likely process for the slow change in warfarin structure.

The stability of warfarin epimers in solution is thought to be governed by the positions of the phenyl and methyl groups on the pyran ring in the cyclic hemiketal [16]. The predicted stabilities of these epimers were compared by optimizing their molecular geometry and using molecular mechanic calculations with a variety of dielectric constants (κ) to mimic several solvents. For an aqueous solution ($\kappa = 78$), one epimer was found to be significantly more stable than the other, with energies of 29.4 and 32.5 kcal/mol, respectively (*Note*: in these calculations, compounds with smaller minimized energies are more stable, with any difference greater than 1 kcal/mol representing a significant change in stability). This difference in stability was found to have a direct relationship with the dielectric constant of the solvent. In DMSO ($\kappa = 48$), these energies were 29.5 and 32.5 kcal/mol, respectively, and in chloroform ($\kappa = 7$) they were 30.1 and 32.6 kcal/mol. In each case, the most stable epimer was the form with the phenyl and methyl groups in the *trans* positions. This corresponds to the structure shown on the right of Figure 6d. This is the same form as the major hemiketal form identified in previous work as the dominant form of warfarin in DMSO and chloroform [16]. All of this information also suggests that this form was the final structure noted for warfarin (i.e., the 3.42 ppm doublet structure) in phosphate buffer and in a 50:50 mixture of DMSO and phosphate buffer.

3.4. Effects of warfarin stability on protein binding

Once the stability of warfarin in aqueous solution had been studied, the effect of this stability on the binding of warfarin to HSA was considered. This was accomplished by examining the retention of warfarin in both freshly prepared solutions and those which had been treated to give greater than 95% conversion of warfarin into its final form (i.e., storage at 45 °C for 4 days). Typical chromatograms obtained in these experiments for injections of *R*-warfarin are shown in Figure 8; similar results were seen for *S*-warfarin. The retention factors that were measured for *R*- and *S*-warfarin are summarized in Table 2.

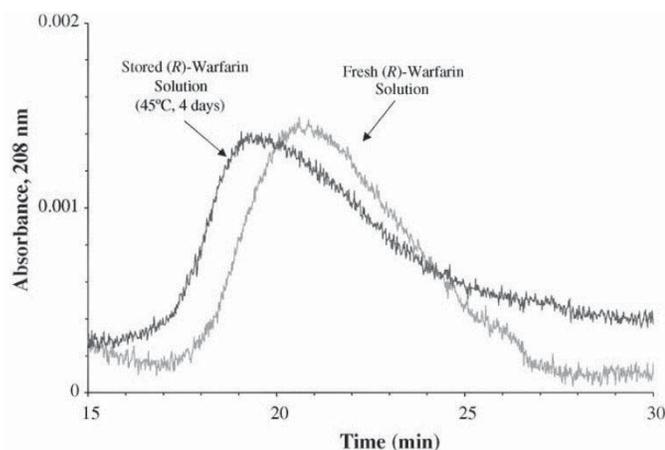


Figure 8. Chromatograms showing the change in retention times on an immobilized HSA column for the original and converted forms of *R*-warfarin at 37 °C. The black line indicates the converted warfarin and the gray line is the unconverted warfarin. Similar results were obtained for *S*-warfarin (data not shown).

Table 2. Retention factors measured at various temperatures for fresh and stored solutions of *R*- and *S*-warfarin on an immobilized HSA column

Type of analyte and solution ^a	Retention factors measured at several column temperatures ^b		
	10 °C	25 °C	37 °C
<i>R</i> -warfarin			
Fresh solution	41(±1)	32.4(±0.3)	26.9(±0.2)
Stored solution	36(±1)	31.6(±0.3) ^c	23.0(±0.5)
<i>S</i> -warfarin			
Fresh solution	62(±1)	N.D.	31.0(±0.1)
Stored solution	60(±1)	N.D.	29.0(±0.3)

a All solutions were prepared in pH 7.4, 0.067 M phosphate buffer using the pure enantiomeric forms of warfarin. The fresh solutions were used within 1 day of preparation and stored at 4 °C in between injections on the chromatographic system. The stored solutions were used after being kept at 45 °C for 4 days.

b The numbers in parentheses represent a range of 1 standard deviation of the mean for three injections. N.D.: not determined.

c The smaller shift noted for *R*-warfarin at 25 °C vs. 10 or 37 °C may be due to differences in the contributions of changes in entropy versus enthalpy in the binding of the original and final forms of the warfarin at these temperatures, as has been noted when comparing the binding of *R*- and *S*-warfarin to HSA [10].

It was found that *R*-warfarin had statistically significant changes in the retention factors measured for the fresh and stored warfarin samples. A decrease was seen in retention of 14.5% for binding studies conducted at 37 °C, a decrease of 2.5% at 25 °C, and a decrease of 12.2% at 10 °C. Most of these shifts were much larger than the run-to-run variation noted in warfarin retention, which ranged from 0.3 to 2.8% (average, 1.3%) over three injections. Furthermore, it was determined from control experiments that these shifts were not due to changes in the HSA column, since these studies were performed over a short period of time compared to the usable lifetime of this column (i.e., 4 days and 20–30 injections versus a usable lifetime of 9 months and over 500 injections) [30]. Thus, it was concluded that this shift in retention was related to the change in warfarin structure that was noted earlier in the fresh and stored solutions. The measured retention factors for *S*-warfarin were statistically similar for both the stored and fresh solutions. The only difference in retention factors was seen at 37 °C when the retention factor for *S*-warfarin decreased by approximately 6.5% during long-term storage when used in binding experiments performed at 37 °C. These differences between *R*- and *S*-warfarin can be attributed to the position of warfarin in the binding pocket of HSA. *R*-warfarin has previously been reported to interact mainly with the interior of the binding site, while *S*-warfarin interacts to a greater degree with the site's outer surface [10]. Due to the orientation of *R*- and *S*-warfarin in the binding pocket, a conformational change of *R*-warfarin would affect its binding to HSA to a greater extent than for *S*-warfarin.

Although warfarin exists mainly as a cyclic hemiketal in solution, it has been suggested previously that the active form of warfarin which inhibits Vitamin K-dependent clotting factors is the open chain form [17]. Furthermore, a crystal structure of the HSA–warfarin complex indicates that warfarin binds to this protein in an open chain form [28]. Since earlier data in this current report indicates that the original and final structures of warfarin in aqueous solution are mainly its cyclic forms, this means warfarin must undergo an enol–keto tautomerism to create its open chain form for proper binding to HSA. This fits with prior kinetic data indicating that the rate limiting step in warfarin/HSA binding is a conformational change in these species as they form their final drug–protein complex [31]. This model also fits the shifts in retention

seen in Figure 8 and listed in Table 2 since an alteration in the solution-phase form of warfarin would thus affect its observed equilibrium constant for binding to HSA.

While the differences in binding and retention noted here between the fresh and stored forms of warfarin is not drastic, it could have some effect on drug-binding studies when using warfarin as the analyte or displacing agent. This effect was estimated using the data in Table 1 and Table 2. For instance, a solution of *R*-warfarin that is stored for 2 weeks at room temperature (i.e., giving 94% conversion), would be expected to give a 14% decrease in retention and/or binding strength for this drug with HSA. The same solution stored for 2 days (i.e., 34% conversion), would give a decrease of 9% in retention at 37 °C. Although this shift is relatively minor, it is significant compared to the precision of retention times and equilibrium constants that can be obtained using HPLC-based HSA columns (e.g., see data in Table 2 and review provided in Ref. [32]).

These predicted changes in warfarin binding may explain some of the variation that has been noted in previously measured equilibrium constants for *R*-warfarin with HSA. As an example, literature values for the association equilibrium constants between HSA and *R*-warfarin in pH 7.4, 0.067 M phosphate buffer have ranged from $(2.1 \text{ to } 3.3) \times 10^5 \text{ M}^{-1}$ at 37 °C [10, 20, 33–36]. Part of the variation in these numbers may be due to differences in the experimental errors of the various techniques used in these studies (i.e., zonal elution [10, 20, 33], equilibrium dialysis [35, 36], and frontal analysis [34]). However, the results of this report suggest that part of this variation could also be a result of differences in the way the warfarin solutions were used and stored in these studies. Thus, future work in this area should include more detailed information on the storage and usable lifetimes of warfarin solutions that are employed in drug–protein binding studies and other bioanalytical measurements.

4. Conclusions

It was found in this study that warfarin undergoes a slow first-order conversion in aqueous solutions, as demonstrated by spectral changes observed using ^1H NMR spectroscopy. The rate of this reaction increased with temperature, with an average rate constant at pH 7.4 of 0.0086 h^{-1} at 25 °C and 0.041 h^{-1} at 37 °C. It was concluded from further NMR studies and molecular modeling that this process most likely involved the conversion from the minor cyclic hemiketal form of warfarin to its major cyclic hemiketal. This reaction had a small but measurable effect on the binding of *R*-warfarin to HSA, as demonstrated by HPLC using an immobilized HSA affinity column. This change in structure may account, in part, for some of the variation that has been seen in literature values for the binding constants of warfarin with HSA. It was also possible from these results to determine which storage conditions should be used to minimize such effects in future work. These guidelines should help provide more consistent equilibrium constant estimates for this system and greater precision in experiments that use warfarin as a probe or displacing agent in drug binding studies with HSA.

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