Biochemical Pharmacology, Vol. 32, No. 2, pp. 281–285, 1983.
Printed in Great Britain.

0006-2952/83/020281-05 $03.00/0

CONSEQUENCES OF THE N–B TRANSITION OF ALBUMIN FOR THE BINDING OF WARFARIN IN HUMAN SERUM

WILLEM F. VAN DER GIESEN* and JAAP WILTING
Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Utrecht, Catharijnesingel 60, 3511 GH Utrecht, The Netherlands

(*Received* 3 *February* 1982; *accepted* 30 *July* 1982)

Abstract—The protein binding of warfarin in serum has been studied by means of circular dichroism and equilibrium dialysis. Evidence was found that the N–B transition of albumin, occurring around physiological pH, takes place not only in solutions of pure albumin but also in serum. The protein binding of warfarin in serum is pH-dependent and increases with pH especially around physiological pH. This pH-dependent serum binding of warfarin can be reasonably explained by the N–B transition of albumin.

The effect of Ca^{2+} and Mg^{2+} on the protein binding of warfarin in serum is negligible at pH 7.4, whereas at this pH Cl^- increases the free-warfarin concentration by a competitive displacement.

The binding of drugs to plasma proteins can have important consequences for the intensity of biological activity and for the pharmacokinetic behaviour of drugs, especially when the affinity of the drugs to the plasma proteins is high and their volume of distribution is small [1–9].

Albumin† plays a dominant role in the protein binding of drugs in serum. It was found that around physiological pH a conformational change occurs in the albumin molecule [10]; this change is called the neutral-to-base or N–B transition. This N–B transition is affected by physiologically important ions such as Cl^-, Ca^{2+} and Mg^{2+} [11–14], which makes the equilibrium between the N and B forms very sensitive to small pH changes. Sudlow *et al.* [15, 16] and later Sjöholm *et al.* [17] established that the binding of drugs to serum albumin occurs mainly at two distinct sites, the so-called warfarin site (site I) and the diazepam site (site II). The binding of both warfarin and diazepam is sensitive to the N–B transition [11, 14] and it can therefore be expected that the binding of other drugs to site I or II will also be sensitive to this conformational change. This means that the binding of many drugs to albumin will be pH-dependent and also sensitive to the presence of Cl^-, Ca^{2+} and Mg^{2+}. Moreover the effect of these ions on the binding could be a competitive displacement. Values of free-drug concentrations in serum determined by means of equilibrium dialysis were therefore expected to be dependent on the pH of the serum samples and also on the composition of the dialysis fluid [12]. Preliminary experiments in our laboratories confirmed this for warfarin in serum. Gier *et al.* [18] found that the composition of the dialysis buffer also plays an important role in the determination of free concentrations of diazepam in serum by means of equilibrium dialysis. Therefore the free-drug concentrations in plasma or serum reported in literature may differ from the free-drug concentrations *in vivo*.

The existence of the N–B transition of albumin was established only in solutions of pure albumin, and so far no experimental support has been given for the occurence of this conformational change of albumin in its natural environment.

The purpose of this paper is to elucidate whether or not the N–B transition of albumin occurs in serum. Since we [11–13] have prior experience with the binding of warfarin to albumin and since it is known that in serum warfarin is bound exclusively to albumin [19] we chose warfarin as a tool to study the N–B transition of albumin in serum.

MATERIALS AND METHODS

Serums A and B were prepared from pooled human blood from two groups of three healthy volunteers. Serum was used instead of plasma because anticoagulant additives such as citrate or heparin may interfere with the binding of warfarin to serum proteins. The albumin concentrations in both serums was determined by the bromocresol green method [20] and was found to be 3.7 ± 0.1%.

Sodium warfarin [British Pharmacopoeia quality (Brocacef, Maarssen, The Netherlands)] was used without further purification. All other chemicals were of analytical grade (Merck, Darmstadt, F.R.G. or J. T. Baker, Deventer, The Netherlands).

CD studies of the warfarin–protein interaction in serum were carried out using a Dichrograph III (Jobin Yvon, Long Jumeau, France); sensitivity = $1 \times 10^{-6}\,\Delta E \cdot mm^{-1}$, which corresponds with an observed ellipticity of 8.25 mdegrees full scale; time constant = 10 sec; path length = 2 mm. Before warfarin was added to the serum, the latter was passed through a series of membrane filters with pore sizes 0.65, 0.45 and 0.22 μm [types DA, HA and GS (Millipore, Etten-Leur, The Netherlands)]. Observed ellipticities (θ_{obs}s) are the differences between the

* Author to whom correspondence should be addressed.
† Albumin here means human serum albumin.

CD signal of the warfarin–serum mixture and the serum alone at 305–380 nm. Molar NaOH was used to raise the pH of the serum. NaOH can be used to adjust the pH without disturbing the binding, because Na^+ ions do not influence the warfarin–albumin interaction [11]. HCl and H_3PO_4, both at molar concentration, were used to lower the pH of the serum; the use of these two acids is discussed later. The obtained CD signals were corrected for the volume changes due to the addition of alkali or acid.

Free concentrations (c_{free}s) of warfarin were obtained by means of equilibrium dialysis done on a Dianorm equilibrium dialyzer (Diachema A.G., Rüschlikon, Switzerland) with cells each consisting of a 1-ml compartment for the protein solution and a 5-ml compartment for the dialysis fluid. The use of the 5-ml compartment for the dialysis fluid is necessary to obtain a reasonable amount of free warfarin for the accurate determination of c_{free}. Dialysis membranes of hydrated cellulose were used (Diachema type 10.14, mol. wt cut-off of 5000). The c_{free} in the dialysate was determined using high-performance liquid chromatography. Full details of the equilibrium dialysis and analysis procedure have been given previously [11]. Molar NaOH was used to raise the pH of the serum samples. To lower the pH both molar H_3PO_4 and molar HCl were used to investigate whether either of these acids has an effect on warfarin binding in serum.

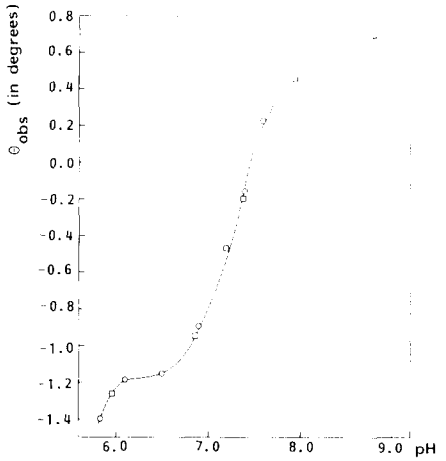

Fig. 1. θ_{obs} of warfarin in serum A at 310 nm as a function of pH at 25°. Warfarin concentration = 1.2×10^{-4} M. Optical pathlength = 2 mm. Molar NaOH was used to raise the pH of the serum. To acidify the serum molar HCl (○) and molar H_3PO_4 (□) were used.

RESULTS AND DISCUSSION

Evidence for the N–B transition of albumin in serum

CD experiments were carried out to find out whether there was a similarity between the pH-dependent induced ellipticity of the warfarin–albumin complex in serum and this ellipticity in solutions of pure albumin. Fig. 1 shows a θ_{obs}–pH profile of a solution of warfarin in serum. No difference can be seen between the results obtained using hydrochloric acid and those using phosphoric acid. The obtained θ_{obs}–pH profile originates from the warfarin–albumin complex since it is known that in serum warfarin is bound exclusively to albumin [19], so other warfarin–protein complexes cannot be involved. This is further supported by the observation that the extrinsic Cotton effects generated by the binding of warfarin in albumin solutions and in serum (not shown) are identical; the maximum CD signal is at 310 nm. The shape of the θ_{obs}–pH profile in Fig. 1 clearly resembles the θ_{obs}–pH profile obtained from warfarin in solutions of pure albumin in the presence of a physiological amount of Cl^- and Ca^{2+} or Mg^{2+} [11, 13]. It has been shown that the profiles obtained from the different solutions of pure albumin originate from the N–B transition that occurs in the albumin molecule in the pH 6–9 range [11].

Previously [10, 11] the N–B transition of albumin has been characterized in terms of the pH_{50} and Hill coefficient [21, 22]. The pH_{50} is defined as the pH where 50% of the albumin is in the B conformation and the Hill coefficient is a measure of the cooperative nature of the N–B transition of albumin. It was found that both the pH_{50} and Hill coefficient are dependent on the composition of the albumin solution. Physiologically important ions such as Ca^{2+}, Mg^{2+} and Cl^- and also certain drugs affect these parameters. The values of the pH_{50} and Hill coefficient as derived from the θ_{obs}–pH profile in Fig. 1 are given in Table 1 together with those for pure albumin. From this table it can be seen that the values for the Hill coefficient and pH_{50} for albumin in serum are not significantly different from the values for these parameters for pure albumin in the presence of Cl^- and Ca^{2+} or Mg^{2+}. Obviously endogenous substances present in serum, other than these ions, are not able to influence significantly the

Table 1. Some characteristic data of CD–pH profiles of warfarin in a pure albumin solution and in serum

Conditions	r	Hill coefficient	pH_{50}	Reference
4.0% HSA in 125 mM NaCl + 5 mM Ca^{2+} or 10 mM Mg^{2+}	0.2	1.4	7.2	13
Serum A	~0.2	1.6	7.3	

r = ratio of warfarin to albumin; pH_{50} = midpoint of the CD–pH profile, i.e. the pH where 50% of the albumin is in the B conformation. The S.D. of the values given for the Hill coefficient and pH_{50} is 0.1.

values of the Hill coefficient and pH_{50}, whereas some exogenous substances alter the values of these parameters considerably. This will be illustrated by two examples: diazepam in the presence of 0.1 M Cl^- forces the N–B equilibrium towards the B conformation giving a pH_{50} of 6.0 and a Hill coefficient of 2.8 [14]; oxyphenbutazone favours the N conformation giving a pH_{50} of 8.8 and a Hill coefficient of 3.0 [23]. If one takes into account how sensitive the Hill coefficient and pH_{50} can be to changes in the nature of substances which are bound to albumin it is reasonable to conclude that the N–B transition of the albumin molecule takes place not only in solutions of pure albumin but also in serum, and that the cooperativity of the N–B transition in serum does not differ from the cooperativity of this transition in solutions of albumin which contain Cl^- and Ca^{2+} or Mg^{2+}.

Effect of pH on the binding of warfarin to albumin in serum

In order to find out whether the pH-dependent value of θ_{obs} is accompanied by a pH-dependent binding of warfarin in serum, c_{free} values of warfarin were determined at different pHs by means of equilibrium dialysis. The results are shown in Fig. 2A. It can be seen that c_{free} is pH-dependent around physiological pH. When the obtained c_{free}–pH profile is compared with the c_{free}–pH profile of warfarin in solutions of pure albumin as presented in Ref. 11 one can see a clear resemblance between them. If the pH-dependent binding of warfarin in serum is due solely to the N–B transition, then the c_{free}–pH profile can be calculated from the c_{free} values at pH = 6 and 9 and the θ_{obs}–pH profile in the way described previously [11]. The result of such a calculation is the drawn curve in Fig. 2A. In view of the complicated composition of serum, it is clear from Fig. 2A that there is good agreement between the experimental and the calculated c_{free}–pH profile. Therefore it can be concluded that in serum the N–B transition of albumin governs the pH-dependent binding of warfarin.

The observed difference in the c_{free} at the extreme pH values (Fig. 2A) needs further attention. The increase in the c_{free} is about 50% when the pH is lowered from 9 to 6. In earlier studies involving solutions of pure albumin to which physiological amounts of Ca^{2+} and Cl^- had been added and where the drug-to-albumin ratio was the same as in this study, we found an increase of 200–300% in the c_{free} when the pH was lowered from 9 to 6 [11]. To facilitate a comparison of the results obtained with pure albumin and serum we calculated the apparent binding constant (K) of warfarin to albumin, assuming albumin to be the only binding component of warfarin in serum [19]. Since the drug-to-albumin ratio is low, K is approximately equal to ν/c_{free} [11] (ν being the number of drug molecules bound per protein molecule). If K_N and K_B are the binding constants for the drug to albumin in the N and B conformations respectively, we obtain the following results: $K_N = (2.6 \pm 0.1) \times 10^5\ M^{-1}$ and $K_B = (4.3 \pm 0.2) \times 10^5\ M^{-1}$ in serum, and $K_N = (2.0 \pm 0.1) \times 10^5\ M^{-1}$ and $K_B = (5.4 \pm 0.2) \times 10^5\ M^{-1}$ in the presence of physiological amounts of Cl^- and Ca^{2+}. These results show that in serum at low pH the affinity of albumin for warfarin is higher than it was in the former study [11] in which pure albumin was used in the presence of physiological amounts of Cl^- and Ca^{2+}. However, in serum at high pH the affinity of albumin for warfarin is found to be lower than in the former study. Cl^- and Ca^{2+} are known to influence the affinity of albumin for warfarin. Because the Cl^- and the Ca^{2+} concentrations used in this study are about the same as they were in the former study and, since in both studies the molecular ratio of these ions to albumin is rather high, the number of ions bound per albumin molecule is about the same in both studies, despite the fact that the albumin concentrations are different. It is reasonable to expect that the degree to which the affinity of albumin for a drug is influenced by an ion (or a neutral compound) will depend on the number of ions bound per protein molecule. Therefore the differences between the albumin concentration in the present (5.5×10^{-4} M) and the earlier study (6×10^{-5} M) cannot be responsible for the fact that

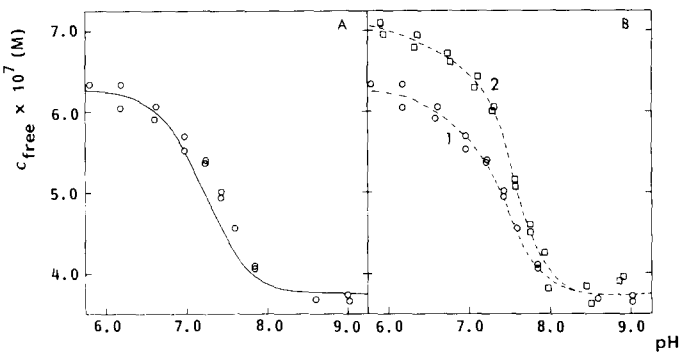

Fig. 2. Free concentrations of warfarin in serum A as a function of pH at 25°. Total warfarin concentration = 9×10^{-5} M. Equilibrium dialysis was used with 0.1 M NaCl as a dialysis fluid. Molar NaOH was used to raise the pH of the serum. In part A of this figure molar H_3PO_4 was used to acidify the serum. The drawn curve was calculated from the θ_{obs}–pH profile in Fig. 1 (see text). In part B of this figure molar H_3PO_4 (○) and molar HCl (□) were used to acidity the serum. The dashed lines are based on the experimental results.

the values of K_N and K_B for warfarin in serum deviate from the values obtained when pure albumin is used in the presence of physiological concentrations of Cl^- and Ca^{2+}. We think that the so-called endogenous binding inhibitors in blood exert different influences on the N and B conformations of albumin, resulting in an increase in K_N and a decrease in K_B. Sjöholm *et al.* [24] were the first to focus attention on endogenous binding inhibitors and to point to the increased concentrations of these compounds in the blood of uremic patients. Recently McNamara *et al.* [25] found evidence that indican and hippuric acid can be considered as being endogenous binding inhibitors.

Effect of Cl^-, Ca^{2+} *and* Mg^{2+} *on the binding of warfarin in serum*

Previously [11, 13] it has been pointed out that the physiologically important ions such as Cl^-, Ca^{2+} and Mg^{2+} affect the warfarin binding to albumin at physiological pH. The effect of Cl^-, Ca^{2+} and Mg^{2+} on the binding of warfarin to albumin in serum at pH = 7.4 has been studied by means of equilibrium dialysis. To examine the effect of chloride on the warfarin binding in serum, serum B containing warfarin was dialysed against a phosphate buffer and a sodium chloride solution. As can be seen from Table 2, the use of a sodium chloride solution as the dialysis fluid results in a c_{free} value that is clearly higher than when a phosphate buffer is used. Previously [12] we found that at a warfarin-to-albumin ratio of 0.1 in the absence of Cl^- the free-warfarin concentration is about 60% lower than in the presence of 0.1 M Cl^-. It was also found that Cl^- and warfarin compete for the first warfarin binding site on the albumin molecule at pH = 7.4. If one assumes that the same simple competitive mechanism operates in serum and that albumin is the only warfarin-binding component [19] one can calculate the effect of Cl^- on the c_{free}. It should be realized that when a phosphate buffer is used as the dialysis fluid the final Cl^- concentration is about 17 mM, because 1 ml serum is dialysed against 5 ml buffer. With a binding constant of 20 M^{-1} for Cl^- on the warfarin binding site [12] it can be estimated that there is likely to be a decrease of about 50% in the c_{free} when a phosphate buffer is used as the dialysis fluid instead of 0.1 M Cl^-. However a decrease of about 15% was found. The fact that this decrease is so much smaller than expected must be due mainly to an impaired affinity of albumin in serum for warfarin in the absence of Cl^-. This conclusion is based on a comparison of the c_{free} values found in serum and in solutions of pure albumin to which Cl^- and Ca^{2+} may have been added [11]. The small Cl^- effect in serum is probably caused by the presence of endogenous binding inhibitors. The Cl^- effect is demonstrated in another way in Fig. 2B. Curve 1 is obtained by using phosphoric acid to adjust the pH and hydrochloric acid is used for curve 2. The latter results in an increasing Cl^- concentration with decreasing pH. As a result of the added hydrochloric acid the final Cl^- concentration at pH = 5.8 is about 130 mM. Below pH = 8 curves 1 and 2 diverge, due to the competition between Cl^- and warfarin and also to the effect of Cl^- on the N–B transition at pHs other than 7.4 [11, 12].

The effect of physiological concentrations of Ca^{2+} and Mg^{2+} can only be studied by means of equilibrium dialysis in the presence of considerable amounts of other ions: the physiological concentrations of Ca^{2+} and Mg^{2+} alone are too low to give an ionic strength of 0.1, which is a minimum value for the ionic strength in the dialysis fluids to reduce the Donnan effect on the c_{free} determination to an acceptable level (<3–4%). Serum already contains 0.1 M Cl^-, and the sodium ion has no effect on the binding of warfarin to albumin. Therefore we used sodium chloride in the dialysis fluids at the physiological concentration of 0.1 M. Calcium chloride and/or magnesium chloride were added to the dialysis fluid in the desired amounts. It can be seen from Table 2 that the effect of Ca^{2+} and Mg^{2+} on the binding of warfarin in serum cannot be ascertained significantly. Previously [11] we found that the free-warfarin concentration in an albumin solution in the presence of 0.1 M Cl^- is reduced by 15–20% when Ca^{2+} is added to a concentration of 2.5 mM. Obviously the Ca^{2+} effect on the albumin binding of warfarin is overruled in serum, probably as a result of the action of endogenous binding inhibitors. The effect of 10 mM Mg^{2+} on the warfarin–albumin interaction at pH = 7.4 in an albumin solution can be neglected, as was found earlier [13], and there is no reason why the effect of 1 mM Mg^{2+} should be larger in serum. It is obvious now that phosphoric acid can be used to acidify serum, as was done to obtain curve 1 in Fig. 2A, without interfering with the binding behaviour of albumin, because the binding of Ca^{2+} and

Table 2. Observed free-warfarin concentrations in human serum determined by means of equilibrium dialysis using various dialysis fluids

Dialysis fluid	c_{free} ($\times 10^7$ M)			
	Serum A	*n*	Serum B	*n*
Phosphate buffer ($I = 0.1$)			3.13 (± 0.09)	3
NaCl	3.72 (± 0.14)	7	3.60 (± 0.13)	3
NaCl + $CaCl_2$	3.61 (± 0.11)	9		
NaCl + $MgCl_2$	3.50 (± 0.06)	8		
NaCl + $CaCl_2$ + $MgCl_2$	3.65 (± 0.18)	6		

Total warfarin concentration = 6×10^{-5} M, pH = 7.4, 25°. Physiological concentrations of NaCl, $CaCl_2$ and $MgCl_2$ were used (0.1 M, 2.5 mM and 1.0 mM respectively). The S.D. is given in parentheses; *n* = number of determinations.

Mg^{2+} to phosphate does not influence the binding of warfarin to albumin in serum within experimental error.

Final remarks

In blood the sensitivity of the free concentration of a drug to the state of the N ⇄ B equilibrium is dependent on the difference in the affinity of that drug for the N and B conformations of albumin. If there is a large difference in the affinity of a drug for albumin in the N and B conformations (K_N and K_B respectively), one should take into account possible effects of acidosis and alkalosis on the c_{free} of that drug in blood. A pH change of 0.1 units in the blood is sufficient to cause a considerable shift in the N ⇄ B equilibrium, and it may then be necessary to adjust the dosage of the drug. From this study it is clear that the difference between K_N and K_B for warfarin in serum is not so large that it is of direct therapeutic importance. However for the determination of free-warfarin concentrations in serum it is important that the pH of the serum samples is near the physiological value. Furthermore when equilibrium dialysis is used the dialysis fluid should contain the physiological amount of Cl^-. A detailed study of the consequences of the N–B transition of albumin in serum for the determination of the c_{free} of warfarin in blood and a discussion about the determination of the c_{free} of drugs other than warfarin have been published elsewhere [26].

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