Effect of Protein Binding on High Performance Liquid Chromatography Analysis of Drugs with an Internal-Surface Reversed-Phase Silica Column

Akimasa SHIBUKAWA,* Terumichi NAKAGAWA, Mamoru MIYAKE, Naohiro NISHIMURA, and Hisashi TANAKA

Faculty of Pharmaceutical Sciences, Kyoto University, Yoshidashimoadachi-cho, Sakyo-ku, Kyoto 606, Japan. *Received September 24, 1988*

The effect of protein binding on the high performance liquid chromatography (HPLC) elution profile of drugs injected directly onto an internal-surface reversed-phase (ISRP) silica column was investigated. When a relatively large volume ($\geq 100\,\mu l$) of the sample solution containing warfarin and bovine serum albumin (BSA) was applied directly onto the ISRP column and elution was carried out with the mobile phase containing an organic modifier, two distinct peaks, both due to warfarin, appeared separated from the protein peak. The peak splitting was not observed in the case of antipyrine–BSA mixed solution, where the protein binding is weak. It was found that the drug bound at the strong-binding site on the BSA molecule was released slowly during the process of chromatography and was eluted as the first peak with a shorter retention time, while the drug bound at the weak-binding site was released quickly, and was eluted as the second peak together with the free drug. The warfarin–BSA interaction at the strong-binding site was evaluated, under minimum influence from the other binding site, from the warfarin concentration obtained from the first peak and the free warfarin concentration determined by the ultrafiltration method.

Keywords warfarin; BSA; drug–protein binding; protein binding; HPLC; internal-surface reversed-phase silica support; Pinkerton column

Introduction

It is well known that a drug in the blood is bound to plasma proteins such as albumin and α_1-acid glycoprotein to a greater or lesser extent and that there is an equilibrium between the concentrations of bound and free species. Studies on drug–protein binding are important, because it affects the pharmacological activities and side effects of the drug as well as the drug distribution and elimination.[1-5] It is known that albumin or α_1-acid glycoprotein binds drug molecules at plural binding sites with different stabilities. These binding sites are usually referred to as strong-binding sites and weak binding sites.

The internal-surface reversed-phase (ISRP) silica support, developed by Pinkerton and co-workers,[6-9] is a new type of packing material for high performance liquid chromatography (HPLC). This support has a hydrophilic stationary phase on the external surface and a moderately hydrophobic stationary phase on the internal surface of pores. As the plasma protein molecules are too large to enter the pores, they are excluded from the column without adsorption, while drugs with small molecular size can enter the pores and be retained on the internal stationary phase. Therefore, this column allows the analysis of drugs in plasma by direct injection without time-consuming pretreatments such as deproteinization and extraction.

The elution profile of a drug injected onto the ISRP silica column together with proteins is governed not only by the retention to the hydrophobic ligand in the pores of the support, but also by the release rate of the drug from protein binding. Therefore, it is of importance to clarify the effect of drug–protein interaction upon the analysis of a drug in protein binding equilibrium following direct injection onto the ISRP silica column. Naturally, the released drug and the free drug initially present in the equilibrium state are both retainable,.whereas the bound drug is excluded together with protein. The release process of the bound drug is influenced by factors such as mobile phase conditions, injection volume and stability of protein binding. It was reported that when a relatively small volume ($\leq 20\,\mu l$) of a plasma sample containing phenytoin,[6] probenecid,[10] lidocaine[10] or cefpiramide,[10] which are known to bind strongly with albumin or α_1-acid glycoprotein, is directly injected into the ISRP column with the mobile phase containing an organic modifier, these drugs are released rapidly and completely from the proteins. These results imply that the free and the released drugs are eluted together to give an apparent single peak with a retention time almost equal to that obtained for protein-free drug solution. However, the effect of the injection volume was not reported in those papers. When a large volume of drug–protein mixed solution is injected onto the ISRP silica column, the release of drug from protein due to sample dilution with the mobile phase is considered to be suppressed. In the case where the drug release proceeds more slowly than the chromatographic partition in the ISRP column, the drug peak is expected to be broadened and finally split, because the peak due to the drug released slowly during the residence in the column will have a shorter retention time than the free drug. In the previous paper,[11] we reported that the injection of a large volume of warfarin potassium (Wf)–bovine serum albumin (BSA) mixed solution suppressed the drug release, and the Wf peak was split. In the present paper, we investigated the effects of stability of protein binding, mobile phase condition and injection volume upon the elution profile of drug–protein mixture in order to clarify the influence of protein binding on the drug analysis by use of HPLC with the ISRP silica column. We also examined the ability of this column to evaluate the interaction between Wf and BSA.

Experimental

Reagents and Materials BSA (fatty acid-free) was purchased from Sigma (St. Louis, Mo.). Wf was manufactured by Eisai Co. Ltd. (Japan). Antipyrine (AP) of guaranteed reagent grade was purchased from Nacalai Tesque Co. (Kyoto, Japan). These reagents were dissolved in potassium phosphate buffer (pH 7.4, ionic strength (I) = 0.17). The concentration of BSA was determined spectrophotometrically using a value of extinction coefficient $E^{1\%}_{1\,\mathrm{cm}}$ of 6.67 at 279 nm. Human plasma was prepared from fresh human blood in a usual manner. The drug–BSA mixed solution was kept 37 °C until applied to the direct HPLC analysis or to the ultrafiltration. Water was purified with a Milli-Q system (Nihon Millipore Kogyo Co., Yonezawa, Japan). Tetrahydrofuran, acetonitrile and isopropanol of

HPLC grade was purchased from Nacalai Tesque Co.

HPLC Conditions Apparatus: An LC-6A system (Shimadzu Co., Kyoto, Japan) equipped with a ultraviolet (UV) detector (SPD-2A, Shimadzu), Rheodyne type 7125 injector and an integrated data analyzer (Chromatopac C-R3A, Shimadzu) was used. Stationary phase: A 5 μm ISPR silica support packed in a 15 or 25 cm × 4.6 mm i.d. stainless steel column (Regis Chemical Co., Morton Grove, IL) was used. This column was a kind gift from Koken Co. (Tokyo, Japan). Column temperature: 37 °C. Detection: UV 313 nm.

Determination of Free Drug Concentration by Ultrafiltration A disposable ultrafiltration kit, Molcut II (UFP1 LGC, Millipore Co.), was used to determine free drug concentration. Adsorption of Wf onto the ultrafiltration membrane was not observed. A 200 μl portion of the filtrate containing free drug was obtained by pressurizing 1 ml of the mixed solution or plasma sample with 10 ml of air. All filtration procedures were performed under 37 °C. A 40 or 80 μl portion of the filtrate was applied to the reversed-phase HPLC to determine the free drug concentration. The HPLC conditions were as follows. Stationary phase: Chemcosorb 7ODSH (15 cm × 4.6 mm i.d., Chemco, Osaka). Mobile phase: potassium phosphate buffer (pH 6.0, I=0.02)/methanol = 1/1 (v/v). Flow rate: 1.5 ml/min. Detection: UV 313 nm.

Results and Discussion

Effect of Stability of Protein Binding upon Drug Elution Profile Figure 1 shows the chromatograms of 50 μM Wf in human plasma. Wf is known to bind strongly to plasma proteins, and the free fraction of Wf in the plasma sample measured by the above-mentioned ultrafiltration method was 1.0%. When a small portion (5 μl) of Wf plasma solution was injected into the ISRP column, Wf gave a single peak (Fig. 1B) with a retention time and peak height almost the same as those observed after injection of the same amount of Wf in buffer solution (Fig. 1A). However, when a large volume (200 μl) of the plasma solution was injected directly into the ISRP silica column, broadening and splitting of the Wf peak were observed (Fig. 1C). Therefore, it must be kept in mind that in the case of direct analysis of plasma samples on an ISRP silica column, the peak height of the drug is not always proportional to the injection volume and a large injection volume may cause peak splitting.

The peak splitting shown in Fig. 1 is considered to be due to the interactions between Wf and endogenous macromolecules, especially plasma proteins such as albumin. The influence of protein binding can be seen clearly by comparing the chromatograms of Wf and AP in BSA solution and buffer. Figure 2 shows the chromatograms of 500 μM Wf in 550 μM BSA solution and in buffer solution, and Fig. 3 shows those of 1 mM AP in 550 μM BSA solution and in buffer solution. Wf is known to bind strongly to albumin, and the free fraction in BSA solution was 2.1%. In contrast, the free fraction of AP in the BSA solution was 91.0%. These values are comparable to the free level of Wf (above mentioned) and AP12) in human plasma. When a small volume (10 μl) of Wf–BSA mixed solution was injected onto the ISRP column, Wf gave a single peak (Fig. 2B) similar to

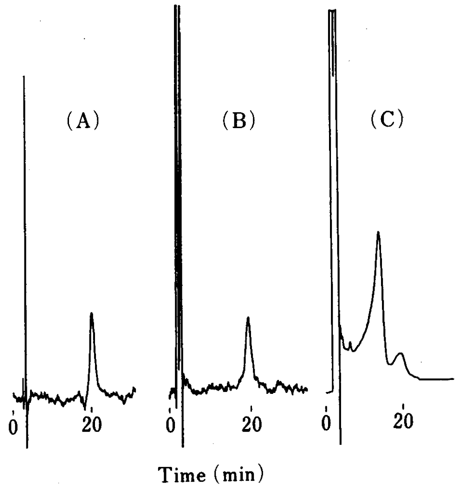

Fig. 1. Elution Profile of 50 μM Wf in Buffer Solution and in Human Plasma

Sample solution: (A) 50 μM Wf in buffer solution (pH 7.4, I=0.17), 5 μl. (B) 50 μM Wf in human plasma, 5 μl. (C) 50 μM Wf in human plasma, 200 μl. Stationary phase: ISRP silica column (25 cm × 4.6 mm i.d.). Mobile phase: potassium phosphate buffer (pH 6.0, I=0.10) containing 5% THF. Flow rate: 1.5 ml/min. Detection: UV 310 nm. The a.u.f.s. of chromatogram (C) is four times larger than that of (A) and (B).

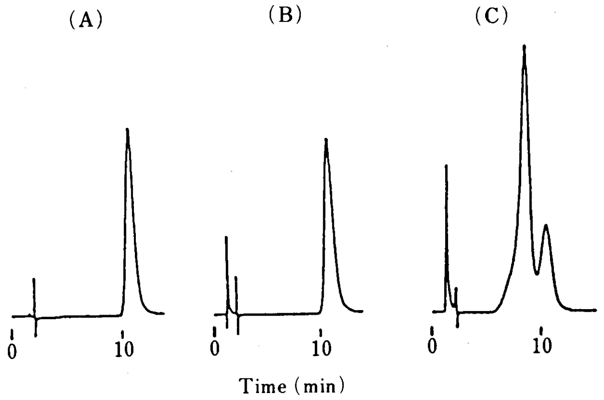

Fig. 2. Elution Profile of 500 μM Wf in Buffer Solution and in 550 μM BSA Solution

Sample solution: (A) 500 μM Wf in buffer solution (pH 7.4, I=0.17), 10 μl. (B) 500 μM Wf in 550 μM BSA solution, 10 μl. (C) 500 μM Wf in 550 μM BSA solution, 200 μl. Stationary phase: ISRP silica column (25 cm × 4.6 mm i.d.). Mobile phase: potassium phosphate buffer (pH 6.0, I=0.10) containing 5% THF. Flow rate: 2.0 ml/min. Detection: UV 310 nm. The a.u.f.s. of chromatogram (C) is eight times larger than that of (A) and (B).

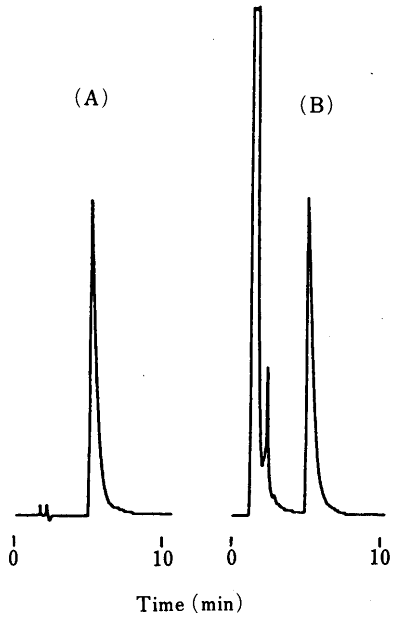

Fig. 3. Elution Profile of 1 mM AP in Buffer Solution and in 550 μM BSA Solution

Sample solution: (A) 1 mM AP in buffer solution (pH 7.4, I=0.17), 200 μl. (B) 1 mM AP in 550 μM BSA solution, 200 μl. Stationary phase: ISRP silica column (25 cm × 4.6 mm i.d.). Mobile phase: potassium phosphate buffer (pH 6.0, I=0.10) containing 2.5% acetonitrile. Flow rate: 1.5 ml/min. Detection: UV 280 nm.

that of Fig. 1B. The retention time and peak height were almost the same as those observed after injection of the same amount of Wf in buffer solution (Fig. 2A). This means that the plasma solution was diluted in the mobile phase, and bound Wf was released rapidly from plasma protein and eluted together with free Wf.

However, when a large volume of the sample solution was injected directly onto the ISRP silica column, the elution profile of the drug changed significantly, depending upon the stability of the protein binding. When 200 μl of the AP–BSA mixed solution was applied (Fig. 3B), AP gave a single peak, and its retention time and peak height were the same as those observed after injection of the protein-free solution (Fig. 3A). On the other hand, when the same volumn (200 μl) of Wf–BSA mixed solution was applied to the colume, broadening and splitting of the Wf peak were observed (Fig. 2C), as in Fig. 1 (C). These results indicate that the large injection volume lowered the degree of dilution of the sample in the mobile phase and, consequently, suppressed the release of the bound Wf from BSA. In contrast, in the case of AP, since the interaction with BSA is weak and the bound AP is considered to be released from BSA more rapidly than Wf, the dissociation of AP–BSA binding was not suppressed even by injection of a large sample volume.

Effect of Mobile Phase on Drug Elution Profile In the practical application of the ISRP silica column to plasma samples, the selection of the mobile phase must be restricted to prevent the denaturation of plasma proteins in the mobile phases which would cause irreversible adsorption of the protein on the ligand, clogging of the column with denatured protein and deterioration of the column efficiency. The recommended conditions are as follows: organic modifiers; $\leq 25\%$ for acetonitrile, $\leq 20\%$ for isopropanol, $\leq 10\%$ for tetrahydrofuran and $\leq 25\%$ for total organic solvent, and pH 6.0—7.5.[7] Within the range of recommended conditions, we investigated the effect of mobile phase conditions upon the elution profile of Wf–BSA mixture applied in a large volume.

Figure 4 shows the chromatograms of 200 μl of Wf–BSA mixed solution injected into the ISRP column. Under the mild mobile phase conditions (pH 7.4, $I=0.17$; also used as the solvent), Wf gave a broad peak incompletely separated from BSA (Fig. 4A), and a plateau region appeared on the chromatogram. It is considered that when drug–protein mixed solution is applied continuously to the ISRP silica column, the concentration of the free drug reaches the steady state in the top region of the column. In this zone, the free drug concentration in the pores is equal to that in the interstice of particles, and the drug and the protein are in the same binding equilibrium state as that in the initial drug–protein mixed solution. After finishing continuous injection of the sample solution, the free drug in this steady state region will be separated from protein and be eluted as a zonal peak with a height corresponding to the free drug concentration. Therefore, the plateau region in Fig. 4A could be due to the elution of the free Wf in this zone, and the plateau height reflects the free Wf concentration of this

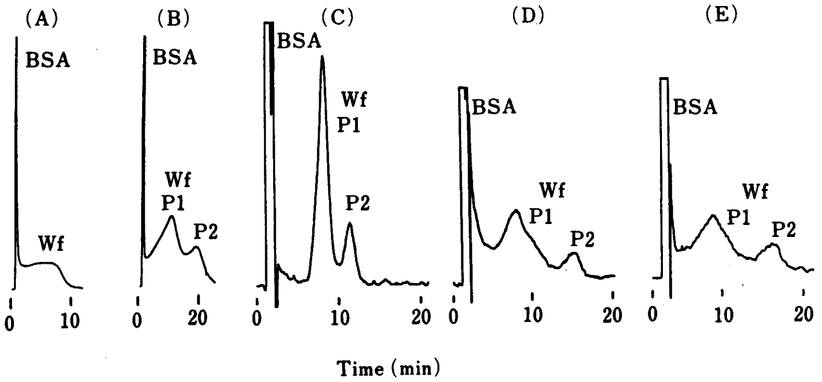

Fig. 4. Effect of the Mobile Phase Condition on the Elution Profile of Wf in BSA Solution

Mobile phase: (A) potassium phosphate buffer (pH 7.4, $I=0.17$); (B) potassium phosphate buffer (pH 6.0, $I=0.10$); (C) (B)+5% THF; (D) (B)+5% acetonitrile; (E) (B)+2.5% isopropanol. Stationary phase: ISRP column (15 cm (A, B) or 25 cm (C—E) × 4.6 mm i.d., Regis Co.). Flow rate: 2.0 ml/min. Detection: UV 310 nm. Sample: (A, B) 200 μM Wf+550 μM BSA, (C—E) 10 μM Wf+550 μM BSA. Injection volume:200 μl.

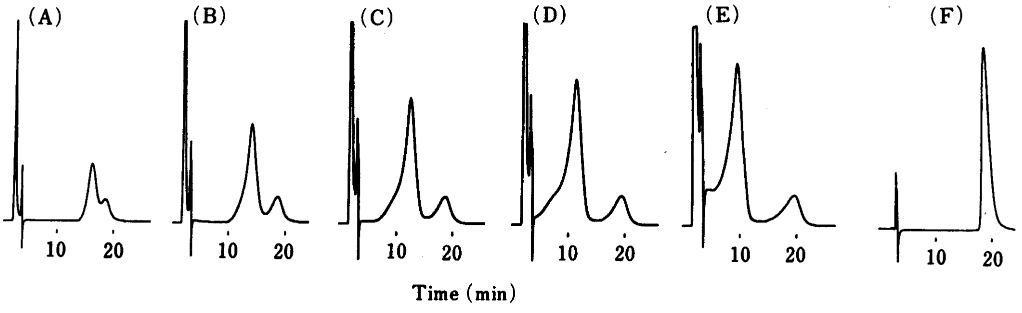

Fig. 5. Effect of Injection Volume on the Chromatograms of 100 μM Wf and 550 μM BSA Mixed Solution

Sample solution: (A)—(E) 100 μM Wf and 550 μM BSA mixed solution; (A) 100 μl, (B) 200 μl, (C) 300 μl, (D) 400 μl, (E) 500 μl. (F) 100 μM Wf in buffer solution (pH 7.4, $I=0.17$), 200 μl. Stationary phase: ISRP silica column (25 cm × 4.6 mm i.d.). Mobile phase: potassium phosphate buffer (pH 6.0, $I=0.10$) containing 5% tetrahydrofuran. Flow rate: 1.5 ml/min. Detection: UV 310 nm.

sample solution. This result indicates the feasibility of using the ISRP silica column to determine free drug concentration by frontal chromatography. The results of detailed investigation of frontal chromatography by use of the ISRP silica column have been reported elsewhere.[13]

When the sample solution was injected under the mobile phase conditions of pH 6.0 and $I=0.10$, the Wf peak was split (Fig. 4B). The splitting of the peak was more apparent when an organic solvent such as tetrahydrofuran, acetonitrile or isopropanol was added to the mobile phase, as shown in Fig. 4C, D, E, respectively. In all cases, the UV-absorption spectra of the split peaks were measured by a photo-diode array detector (MCPD-3500, Otsuka Electronics Co.). They agreed exactly with each other and also with that of BSA-free Wf solution. These results indicate that the release of the drug from the protein was accelerated by the presence of organic modifiers as well as by the changes of pH and ionic strength. The addition of 5% tetrahydrofuran (THF) to the mobile phase used in Fig. 4B resulted in the most distinct separation of the peak (Fig. 4C). Hereafter, the first peak with shorter retention time is designated P1 and the second peak with longer retention time is designated P2.

Effect of Injection Volume on Drug Elution Profile The effect of sample injection volume on the elution profile of warfarin is shown in Fig. 5, where 100—500 μl of 100 μM Wf and 550 μM BSA mixed solution was injected in the mobile phase (pH 6.0, $I=0.10$) containing 5% THF. The Wf peak was split when the sample volume exceeded 100 μl. As the sample volume further increased and hence the sample solution was less diluted with the mobile phase, P1 decreased in retention time and was separated better from P2. In contrast, the retention time of P2 was almost unchanged and equal to that of Wf in the BSA-free solution regardless of sample volume. From these results, it followed that P1 was due to Wf released from BSA in the mobile phase, and P2 comprised free Wf. However, as mentioned later, further investigation revealed that P2 also comprised a part of Wf released from BSA.

The peak areas of P1 and P2 were measured with the integrated data analyzer, and their concentrations (D_1 and D_2, respectively) were determined. As shown in Table I, the sum of D_1 and D_2 values was almost equal to the total Wf concentration (100 μM) regardless of the injection volume. This means that Wf bound to BSA was almost completely released during the residence in the column, and the total concentration could be determined from the combined area of P1 and P2. The D_2 value was constant when more than 200 μl of the sample solution was injected. The injection of too large a volume (more than 400 μl) of the sample solution resulted in the overlap of P1 with the BSA peak, and consequently the total Wf concentration could not be determined.

Table I. The Wf Concentrations of P1 and P2

	Injection volume (μl)				
	(A) 100	(B) 200	(C) 300	(D) 400	(E) 500
D_1 (μM)	94.8	85.4	84.0	—	—
D_2 (μM)	8.64	13.1	13.9	13.6	14.3
D_1+D_2 (μM)	103.4	98.5	97.9	—	—

D_1 and D_2 represent the Wf concentrations corresponding to P1 and P2, respectively. An aliquot of 100—500 μl of 100 μM Wf and 550 μM BSA mixed solution was injected. HPLC conditions were the same as in Fig. 5.

Evaluation of the Interaction between Wf and BSA The D_1 and D_2 values were determined by injection of 200 μl of 550 μM BSA mixed solution containing 10—500 μM Wf. The results are shown in Table II. The low CV (%) indicates good reproducibility of these values. The recovery values of Wf listed in Table II were calculated as the ratio of the sum of D_1 and D_2 values to the total Wf concentration. Wf was recovered completely from BSA solutions. On the other hand, the concentration of free Wf (D_f) of the same sample solution was determined by means of the ultrafiltration method. As shown in Table II, the free Wf concentration of every sample solution was much smaller than the D_2 value. It followed, therefore, that P2 comprised a part of bound Wf in addition to the free Wf.

The interaction of drugs with plasma proteins is of great importance in pharmacokinetic studies. In chemotherapy with antiepileptic agents (*e.g.* phenytoin, carbamazepine and valproic acid), antiarrythmic agents (*e.g.* lidocaine and disopyramide), and many other drugs, it has been pointed out that many factors, such as the change of the concentration or nature of plasma proteins, the displacement of bound drug by simultaneously administered drugs and endogenous inhibitors, and disease and age of patients, influence the drug–protein interaction and vary the free drug level in plasma.[3] If the dispositions of a drug bound at different sites on protein can be evaluated separately, it may offer more detailed information about the pharmacokinetics and pharmacodynamics of the drug. Therefore, the development of a simple and rapid analytical method to evaluate protein binding at different sites is of significance.

It has been reported that one BSA molecule associates with two molecules of warfarin at the strong-binding sites (site I) and five Wf molecules at the weak-binding sites (site II).[14] As mentioned in Fig. 3, the release of the drug which binds weakly to the protein is considered to be very rapid, and the released drug is eluted together with the free drug even when a large volume of sample is injected. Therefore, it is considered that weakly bound Wf was released quickly from weak-binding sites and eluted together with free Wf, and P1 was due solely to Wf released from strong-binding sites. Accordingly, it is expected that analysis with the ISRP

Table II. The Concentrations of P1 (D_1), P2 (D_2) and Free Wf (D_f)

Wf –BSA (μM) (μM)	D_1		D_2		Recovery (%)	D_f	
	(μM)	CV%	(μM)	CV%		(μM)	CV%
500–550	450.7	0.50	52.1	1.82	100.6	10.4	1.25
300–550	272.2	0.39	28.3	1.41	100.2	4.47	6.40
200–550	174.2	1.00	24.7	1.66	99.5	2.44	1.15
100–550	85.4	1.22	13.1	6.34	98.5	1.08	10.2
70–550	62.4	0.69	8.82	5.05	101.7	0.741	3.72
50–550	42.4	3.61	7.48	1.78	99.8	0.510	7.84
20–550	17.3	1.68	3.02	1.49	101.6	0.204	16.2
10–550	8.50	4.81	1.50	7.07	100.0	0.096	19.8

K_1 and K_2 represent the binding constants for site I (strong-binding site) and site II (weak-binding site), respectively. D_f values were determined by use of ultrafiltration (Molcut II, Millipore). CV, coefficient of variation ($n=5$). Injection volume, 200 μl. Other HPLC conditions were the same as in Fig. 5.

silica column may allow us to evaluate drug–protein interactions at different binding sites separately, with the combined use of the ultrafiltration method to determine free drug concentration.

If Wf released from site II was separated completely from Wf released from site I under the present HPLC conditions, D_2 would be the sum of the concentration of free Wf and that of Wf released rapidly from site II, and D_1 would be the concentration of Wf released slowly from site I. Consequently, the binding parameters for each site can be calculated from the Scatchard plot of the data in Table II using Eqs. 1 and 2,

$$D_1/D_f = -K_1 \cdot D_1 + K_1 \cdot n_1 \cdot [\mathrm{BSA}] \tag{1}$$

$$(D_2 - D_f)/D_f = -K_2 \cdot (D_2 - D_f) + K_2 \cdot n_2 \cdot [\mathrm{BSA}] \tag{2}$$

where [BSA] represents the total concentration of BSA (*i.e.* 550 μM), and n_1 and n_2 represent the number of sites I and II on one BSA molecule, respectively.

The resulting values of the binding parameters calculated from Eq. 2 ($\log k_2 = 5.44$ and $n_2 = 0.096$, $R = -0.944$) differed considerably from the reported values obtained by dynamic dialysis experiments ($\log K_2 = 3.21$ and $n_2 = 5.0$).[14] This implies that the concentration of Wf bound at site II was not determined accurately by the present method, probably because P2 partially included Wf molecules released from site I, and the Wf concentration bound at site II was overestimated. On the other hand, the binding parameter values for site I calculated from Eq. 1 ($\log k_1 = 5.00$ and $n_1 = 1.61$, $R = -0.997$) were comparable to the reported results ($\log K_1 = 5.06$ and $n_1 = 2.0$).[14] This indicates that the present method allows us to determine the approximate Wf concentration bound at site I by removing Wf bound at site II from P1, although the actual concentration is considered to be a little higher than the D_1 value. In the usual Scatchard-plot analysis where the sum of drug concentrations bound at both sites is used, the drug–protein interaction at the strong-binding site is estimated not individually but concurrently with that at the weak-binding site, and data for the samples with high drug concentrations are needed in order to estimate the binding at site II. However, the present HPLC method combined with the ultrafiltration method allows the drug–protein interaction at the strong-binding site to be estimated from the data for samples containing relatively low drug concentrations. The protein binding at site I is estimated from the D_1 and D_f values directly, under minimum influence from the other binding site.

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