Communications to the Editor

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ANALYSIS OF WARFARIN-ALBUMIN BINDING BY HPLC WITH INTERNAL-SURFACE REVERSED-PHASE SILICA COLUMN

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Protein binding of warfarin with bovine serum albumin (BSA) was analyzed by HPLC with an internal-surface reversed-phase (ISRP) silica column. When a relatively large volume (≥100 μl) of the sample solution containing warfarin and BSA was applied directly to the ISRP column, two distinct peaks appeared separately from the protein peak. One of these had a short retention time and was ascribable to warfarin released from the strong-binding sites on BSA molecules during the process of chromatography. The other peak had a long retention time and was due to the sum of free warfarin and that released from weak-binding sites. The separation profiles of these peaks were investigated with varying injection volumes and mobile phase conditions. The binding constants for the strong site and the weak site were determined from the area of these warfarin peaks. The results were comparable to the reported values.

KEYWORDS——warfarin; BSA; drug-protein binding; protein binding; HPLC; internal-surface reversed-phase silica support; Pinkerton column; protein binding constant

Internal-surface reversed-phase (ISRP) silica, a new type of packing material for HPLC, 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 moleculars 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. This allows separation of drugs from proteins when plasma samples are directly injected without pretreatment such as deproteinization or extraction. The synthesis,[1] properties, and application[2,3] of the ISRP column were reported previously.

Incorporation of a second kinetic process into the chromatographic partition

system generally prolongs the retention time and broadens the peak of the sample substances involved. The extent of such effects depends clearly on the relative rate of the second process and the partitioning process. The operating conditions, such as the flow-rate of the mobile phase and the injection volume of the sample solution, affect the relative rate. Since the drug-protein binding is in a certain equilibrium state in the plasma sample, the dilution of the plasma sample with the mobile phase, following injection into the ISRP column, breaks the equilibrium. As a result, the amount of unbound drug increases depending on the rate of dissociation of drug-protein binding. For instance, it is known that when a relatively small volume ($\lesssim$20 μl) of a plasma sample containing phenytoin,[1] probenecid,[3] lidocaine[3] or cefpiramide,[3] which are known to bind strongly with albumin or α_1-acid glycoprotein, is directly injected into the ISRP column, these drugs are released rapidly and completely from the proteins. Consequently, the free and the released drugs are eluted altogether to give an apparent single peak with retention time almost equal to that obtained from the protein-free solution. Thus, the total amounts of drug were determined. However, when the drug release proceeds more slowly than the chromatographic partition in the ISRP column, the drug peak is broadened or split, but the peak due to the free drug will have longer retention time than the drug that is released slowly during the residence in the ISRP column. Thus, it is expected that the accelerated release of the drug sharpens the peak with the retention time approaching that of the free drug.

Figure 1 shows the effect of the mobile phase condition on the elution profile of warfarin following injection of 200 μl of buffer solution containing known amounts of warfarin and BSA into the ISRP column. The injection of 200 μM warfarin and 550 μM BSA into the mobile phase (pH 7.4, ionic strength (I) = 0.17) gave a broad peak of warfarin incompletely separated from BSA (Fig. 1 (A)) with a mean retention time of about 6 min, shorter than that obtained from the BSA-free solution (7.2 min). When the pH and ionic strength of the mobile phase were lowered (pH 6.0, I = 0.1), the warfarin peak was split (Fig. 1 (B)). The splitting of the peak was more apparent when an organic solvent was added to the mobile phase, as shown in Fig. 1 (C), (D) and (E). The addition of 5% tetrahydrofuran to the mobile phase used in Fig. 1 (B) resulted in a more distinct separation of the peak (Fig. 1 (C)) compared with mobile phases containing 5% acetonitrile (Fig. 1 (D)) and 2.5% isopropanol (Fig. 1 (E)). The first peak with short retention time is designated P1 and the second peak with long retention time P2. In all cases, the UV-absorption spectra for these peaks measured by a photo-diode array detector (MCPD-3500, Otsuka Electronics Co.) agreed exactly with each other and with that of BSA-free warfarin solution. The retention time of P2 was very close to that of warfarin in the BSA-free solution, while the retention time of P1 varied between those of P2 and BSA

depending on the mobile phase conditions. These results suggest that P1 can be ascribed to warfarin released from BSA, and that the organic modifier accelerated the dissociation of warfarin-BSA binding.

The effect of sample volume on the elution profile of warfarin is shown in Fig. 2, where the mobile phase contained 5% tetrahydrofuran. The peak was split only when the sample volume exceeded 100 µl. As the sample volume increased, P1 decreased

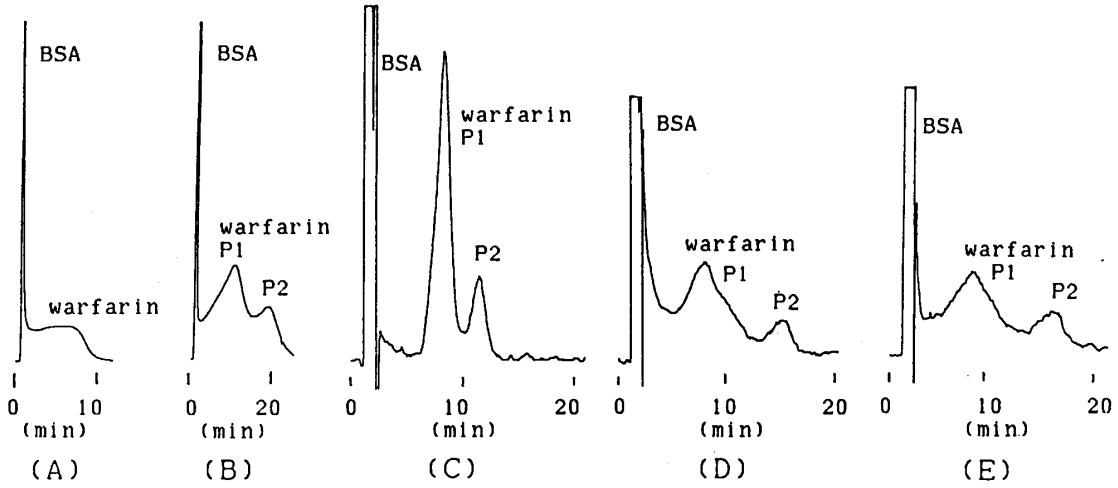

Fig. 1. Effect of the Mobile Phase Condition on the Elution Profile of Warfarin in Bovine Serum Albumin (BSA) Solution. Mobile phase: (A) potassium phosphate buffer (pH 7.4, I = 0.17); (B) potassium phosphate buffer (pH 6.0, I = 0.10); (C) (B) + 5% tetrahydrofuran; (D) (B) + 5% acetonitrile; (E) (B) + 2.5% isopropanol. Stationary phase: ISRP column (15 cm (A,B) or 25 cm (C-E) x 4.6 mm I.D., Regis Co.). Flow rate: 2.0 ml/min. Detection: UV 310 nm. Sample: (A,B) 200 µM warfarin + 550 µM BSA, (C-E) 10 µM warfarin + 550 µM BSA. Warfarin potassium and BSA were dissolved in potassium phosphate buffer (pH 7.4, I = 0.17). Injection volume: 200 µl.

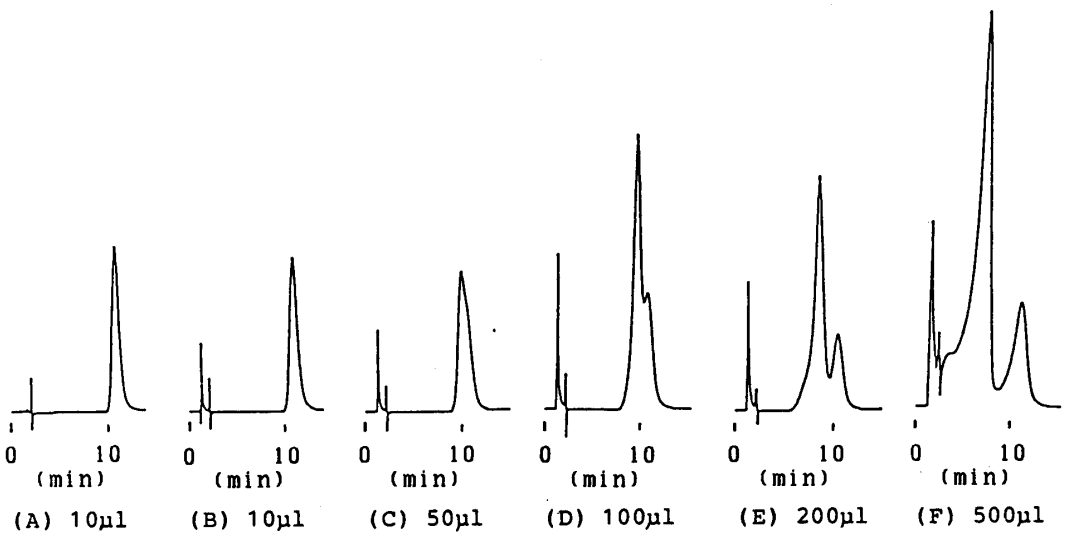

Fig. 2. Effect of Injection Volume on Elution Profile of Warfarin in Buffer Solution (A) and 550 µM BSA Solution (B-F). Sample: 500 µM warfarin + 0 µM (A) or 550 µM (B-F) BSA. Injection volume: 10-500 µl. Other conditions are the same as Fig. 1(C).

in retention time and separated better from P2. The injection of too large a volume (500 µl) of the sample solution resulted in the overlap of P1 with the BSA peak. In contrast, the retention time of P2 was almost unchanged and equal to that of warfarin in the BSA-free solution regardless of sample volume. From these results it appears that a larger volume of sample solution was less diluted with the mobile phase, so that the dissociation of warfarin-BSA binding was suppressed. The peak areas of P1 and P2 were measured with a Chromatopac C-R3A (Shimadzu Co.) and their concentrations were determined. The results indicated that when a 200 µl portion of the sample solution containing 100 µM warfarin and 550 µM BSA was injected repeatedly (n = 5), 85.4 µM of warfarin was recovered in P1 and 13.1 µM of warfarin in P2. This means that the warfarin bound to BSA was almost completely released in the mobile phase, and the total concentration could be determined from the combined area of P1 and P2. On the other hand, the concentration of free warfarin was determined by means of ultrafiltration of the same sample solution followed by HPLC of the filtrate. It was found that only 1.1% of the total amount of warfarin had been free from the binding with BSA. It followed, therefore, that P2 comprised 1.1µM of free warfarin and 12.0 µM of released warfarin. It has been reported that one BSA molecule associates with two molecules of warfarin at the strong-binding sites (site I) and five warfarin molecules at the weak-binding sites (site II).[4] Assuming that P1 is due to warfarin released slowly from site I and P2 is due to the sum of the free warfarin plus that released rapidly from site II, we can calculate the binding constants K1 for site I and K2 for site II. The result, log K1 = 4.87 ± 0.031 and log K2 = 3.54 ± 0.19,[5] were comparable to the reported values obtained by means of dynamic dialysis (log K1 = 5.06 and log K2 = 3.21).[4]

The present results show that it is feasible to use the ISRP column to estimate strong drug-protein binding, by the direct application of plasma samples. It is considered that the release velocity of a drug from protein can be estimated by analyzing the elution profile of the drug. Detailed investigations will be reported in the near future.

REFERENCES AND NOTES

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5) These binding constants (at 37°C) were calculated from the equations

K1 = D1/Df(2 x [BSA]-D1), and K2 = (D2-Df)/Df(5 x [BSA]-(D2-Df)).

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