Determination of Free Drug in Protein Binding Equilibrium by High-Performance Frontal Analysis Using Internal-Surface Reversed-Phase Silica Support

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

Faculty of Pharmaceutical Sciences, Kyoto University, Yoshidashimoadachi-cho, Sakyo-ku, Kyoto 606, Japan. *Received August 25, 1988*

An internal-surface reversed-phase silica column was employed in the frontal analysis method to determine free drug concentration in warfarin (Wf)–bovine serum albumin (BSA) mixed solution and in indometacin(Im)–BSA mixed solution.

When a 4-ml portion of aqueous solution containing 2—30 μM Im and 28 μM BSA and a 10-ml portion of aqueous solution containing 0.5—175 μM Wf and 28 μM BSA were applied, the elution curves reached a plateau level corresponding to both free drug and drug–BSA complex (β-plateau), followed by another plateau due to free drug alone (γ-plateau). The drug concentration at the γ-plateau agreed well with the free drug concentration determined after ultrafiltration of the same solution.

The γ-plateau was observed even when the applied volume was reduced to a level which was insufficient to produce the β-plateau. The injection of a 400-μl portion of the 0.5—100 μM Im and 28 μM BSA mixed solution and a 90-μl portion of 50 μM Im and 550 μM BSA mixed solution onto the ISRP silica column gave a clear γ-plateau. Compared to the conventional frontal analysis, the present method can determine a wide range of drug levels with much smaller injection volume. This method is applicable to plasma. By a single frontal analysis, both free and total concentrations of carbamazepine in plasma were determined simultaneously.

Keywords protein binding; drug–protein interaction; albumin; internal-surface reversed-phase silica column; high performance liquid chromatography; frontal analysis

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 drug–protein binding affects the pharmacologic activities and side effects of the drug as well as the drug distribution and elimination.[1-3] Several methods such as equilibrium dialysis and ultrafiltration have been used to determine the concentration of free drug in drug–protein mixed solutions.[1,2]

The gel filtration frontal analysis method[4] was first applied by Nicol and Winzor[5] to the evaluation of protein–protein interaction, and was used for the quantitative study of drug–protein binding by Scholtan.[6] In this method, a large volume of drug–protein mixed solution (larger than the void volume of the column) is applied continuously to a size-exclusion gel column to achieve a steady-state concentration. The elution profile obtained consists of three zones (α, β and γ-plateau zones). When drug–protein mixed solution is applied to the gel, the bound drug is released immediately from protein in the mobile phase, and penetrates into pores of the gel together with the free drug, while the protein is excluded. Therefore, free protein is eluted first from the column (α zone). After the flow of drug–protein mixed solution in the column has reached the steady-state, they are eluted together and give a maximum plateau level (β-plateau) on the elution curve. Naturally, the concentrations of free and bound drugs in the steady-state are expected to be the same as those in the initial drug–protein mixed solution. After finishing continuous introduction of the drug–protein mixed solution into the gel, the protein concentration rapidly decreases, and finally the drug retained in the pores is eluted. As a result, the β-plateau is followed by a steep descent to another plateau (γ-plateau zone) which represents the free drug concentration in the drug–protein mixed solution.

This method does not suffer the Donnan effects and loss of drug from undesirable adsorption often encountered in the equilibrium dialysis method and ultrafiltration method. However, it has been a defect of this method that a large volume of drug–protein mixed solution is needed to observe a clear γ-plateau. For example, it was reported that 45 ml of salicylate–human serum albumin (HSA) mixed solution[7] and 60 ml of warfarin–HSA mixed solution[8] were used to obtain clear γ-plateaus using a Sephadex G-25 column (25 cm × 1 mm i.d.). Sebille *et al.* employed this method with an high performance liquid chromatography (HPLC) system using a 5—10 μm μBondagel column (30 cm × 3.9 mm i.d.) with 125 Å pore diameter; they required 18 ml of warfarin–BSA mixed solution.[9]

Internal-surface reversed-phase (ISRP) silica, developed by Pinkerton and co-workers,[10,11] is a new type of packing material for HPLC. This support has hydrophilic diol-glycine groups on the external surface and a moderately hydrophobic tripeptide partitioning phase (Gly–Phe–Phe) 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 onto the external surface, while drugs with small molecular size can diffuse into the pores and be retained on the internal partitioning phase. Therefore, this allows separation of drugs from proteins following direct injection of a plasma sample without pretreatments such as deproteinization and extraction. The synthesis, properties, and application of the ISRP column were reported previously.[10-15]

In the case of a hydrophobic drug which is strongly retained on to the ISRP silica column, a short column may allow the determination of free drug concentration by injecting a small volume of the drug–protein mixed solution. In the present paper, the ISRP silica support was employed in the frontal analysis method and used for determinations of the concentrations of free warfarin (Wf)

in Wf–bovine serum albumin (BSA) mixed solution and of free indometacin (Im) in Im–BSA mixed solution. Furthermore, the concentration of carbamazepine (CBZ) in human plasma was determined by this method, and the applicability of the method to plasma was demonstrated.

Experimental

Reagents and Materials BSA (fatty acid free) was purchased from Sigma (St. Louis, Mo.). Warfarin potassium was a gift from Department of Pharmacy, Kyoto University Hospital. Indometacin of guaranteed reagent grade was purchased from Nacalai Tesque (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 extinction coefficient $E^{1\%}_{1cm} = 6.67$ at 279 nm. CBZ of guaranteed reagent grade was purchased from Wako Pure Chemicals (Osaka, Japan). Human plasma was prepared from fresh human blood in a usual manner. An ethanol solution of 10 mM CBZ was spiked into human plasma to prepare a plasma sample containing 8 μM CBZ. The drug–BSA mixed solution and plasma sample were kept at 37 °C until used in frontal analysis or ultrafiltration.

HPLC Conditions for Frontal Analysis Apparatus: A Tri Rotar III system (Jasco, Japan) equipped with a ultraviolet (UV) detector, SPD-2A (Shimadzu, Japan), and an integrated data analyzer, Chromatopac C-R3A (Shimadzu), was used. A Rheodyne type 7125 injector with a 1-, 5- or 10-ml loop was used for sample injection.

Mobile phase: potassium phosphate buffer (pH 7.4, I = 0.17).

Flow rate: 0.3—1.2 ml/min.

Stationary phase: 5 μm ISRP silica support (Regis Chemical Co., Morton Grove, IL) was a gift from Koken Co. (Tokyo, Japan), and was slurry-packed into the stainless steel columns. Column (A), 3.0 × 4.6 cm i.d.; column (B), 10 cm × 4.6 mm i.d. (two 5-cm columns were connected in series).

An ISRP silica column of 15 cm × 4.6 mm i.d. (Regis Chemical Co.) was purchased from Koken Co.

Column temperature: 37 °C, controlled in a water bath.

Detection: UV 313 nm for Wf. UV 315 and 260 nm for Im. UV 300 nm for CBZ.

Determination of Free Drug Concentration by Ultrafiltration A disposable ultrafiltration kit, Molcut II (UFP1 LGC, Milipore Co.), was used as a reference standard method to determine free drug concentration. Wf and CBZ were not adsorbed on the ultrafiltration membrane, whereas Im was adsorbed. Therefore, in the latter case, the membrane were preliminarily saturated with Im by filtration of about 150 μl of the mixed solution. This treatment was effective to obtain reproducible results. Each 200 μl portion of the filtrate containing free drug was obtained by pressurizing 1 ml of the mixed solution with 4 ml of air. All filtration procedures were performed under 37 °C. A 40- or 80-μl portion of the filtrate was subjected 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)/MeOH = 1/1 for Wf, 4/6 for Im. H_2O/MeOH = 1/1 for CBZ.

Flow rate: 1.5 ml/min for Wf and CBZ, 2.0 ml/min for Im.

Detection: UV 313, 225, 280 nm for Wf, Im and CBZ, respectively.

Results and Discussion

Table I shows the relationship between injection volume of drug solution without protein and the peak height of the drug. When more than 3.0 ml of 1 μM Wf or 3 μM Im solution was applied to the ISRP column (A), the peak height reached a maximum level irrespective of the flow rate. This means that sample injection of at least 1 ml per 1-cm length of the ISRP silica column (4.6 mm i.d.) provides the β-plateau on the elution curve.

Figure 1 shows the elution profiles of a 4-ml portion of 30 μM Im and 28 μM BSA mixed solution applied to the ISRP column (A). A clear β-plateau appeared regardless of the flow rate. On the other hand, the shape of the γ-plateau depended significantly on the flow rate. A clear γ-plateau appeared only when the flow rate was 0.3 ml/min. Therefore, the flow rate was fixed at 0.3 ml/min.

In the frontal analysis of Wf–BSA mixed solution, the ISRP silica column (B), which is longer than the column (A), was necessary to obtain a clear γ-plateau, because of the weaker retention of Wf. (The values of capacity factor on the ISRP silica column (A) were 6.22 for Wf, and 12.72

TABLE I. Effect of Injection Volume on Peak Height of Im and Wf

Injection volume	1 μM Wf (k' = 6.22) Flow rate		3 μM Im (k' = 12.72) Flow rate	
	0.3 ml/min	1.2 ml/min	0.3 ml/min	1.2 ml/min
1.0 (ml)	7.65 (cm)	7.45 (cm)	6.02 (cm)	5.42 (cm)
2.0	8.35	8.24	7.29	6.93
2.5	8.48	8.48		
3.0	8.48	8.50	7.46	7.45
3.5			7.46	7.42
4.0	8.50	8.50	7.43	7.45
4.5			7.43	7.45

Stationary phase: ISRP column (A) (3.0 cm × 4.6 mm i.d.). Mobile phase: potassium phosphate buffer (pH 7.4, I = 0.17). Detection: UV 313, 315 nm. Column temperature: 37 °C.

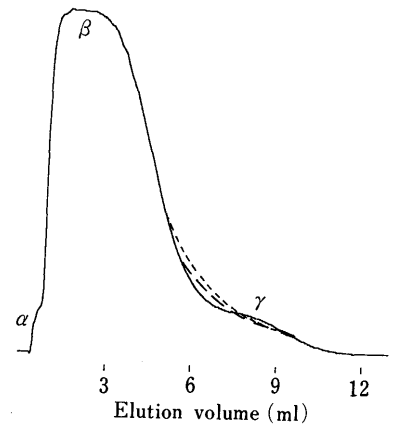

Fig. 1. Effect of Flow Rate on Elution Profile of 28 μM BSA–30 μM Im Mixed Solution

Stationary phase: ISRP column (3.0 cm × 4.6 mm i.d.). Mobile phase: potassium phosphate buffer (pH 7.4, I = 0.17). Flow rate: 0.3 ml/min (——), 0.6 ml/min (– – – –), 1.2 ml/min (------). Detection: UV 315 nm. Column temperature: 37 °C. Injection volume: 4 ml.

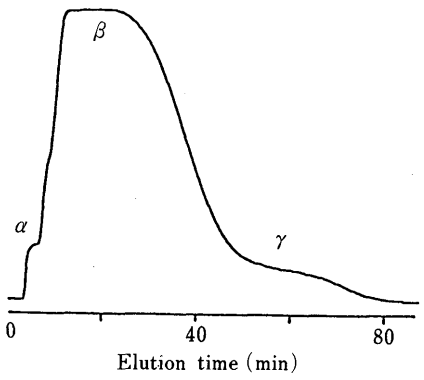

Fig. 2. Elution Profile in Frontal Analysis of 28 μM BSA–10 μM Wf Mixed Solution

Stationary phase: ISRP column (10 cm × 4.6 mm i.d.). Mobile phase: potassium phosphate buffer (pH 7.4, I = 0.17). Flow rate: 0.3 ml/min. Detection: UV 313 nm. Column temperature: 37 °C. Injection volume: 10 ml.

for Im.) Consequently, the injection volume was fixed at 10 ml. A typical elution profile is shown in Fig. 2.

A BSA solution (28 μM) containing 0.5—175 μM Wf or 2—30 μM Im was subjected to this frontal analysis. Table II lists the drug concentration corresponding to the height of the γ-plateau (C_p). Since the plateau was apparently not flat, the height was measured at the inflection point. The calibration lines for the drugs were constructed by applying 4 ml of 0.05—20 μM Im and 10 ml of 0.05—120 μM Wf to column (A) and column (B), respectively. For comparison, the free drug concentration (C_f) was determined by means of the ultrafiltration method. The C_p values of both drugs agreed well with the C_f values.

The profile of the γ-plateau depended on the drug concentration. When a sample solution with high drug concentration (more than 175 μM Wf or 30 μM Im) was applied, the analysis was difficult because the free drug eluted in the descending curve and the γ-plateau disappeared. Furthermore, although the sample volume applied in the present analysis was smaller than that in the conventional frontal analysis using usual size-exclusion gel, analysis with a still smaller injection volume would be desirable.

Krieglstein and Kuschinsky reported that when drug–protein mixed solution was applied to a size exclusion support on which the drug was adsorbed reversibly, the γ-plateau appeared even when the β-plateau was not observed.[16] Therefore, it is expected that the ISRP silica column would allow us to determine free drug concentration with a much smaller injection volume of sample solution than that necessary to obtain the β-plateau.

Figure 3 shows the effect of injection volume upon the elution profile of 10 μM Im–28 μM BSA mixed solution applied to the ISRP silica column (A). As the injection volume was increased, the Im peak broadened toward the elution peak of BSA (Fig. 3h). When 300—400-μl portions of the solution were applied, the Im peak attained the maximum height, and the γ-plateau appeared, although the β-plateau did not appear. When more than 400 μl of the solution was applied, this region was not flat because of the overlap with the BSA peak.

The drug concentration corresponding to the plateau height was determined over a wide range of Im concentration (0.5—100 μM) in the mixed solution, where the

TABLE II. Concentration of γ-Plateau (C_p) and Free Concentration (C_f) of Im and Wf

Sample solution (μM)	C_p (μM) $n=2$	C_f (μM) $n=4$
Im–BSA		
2–28	0.181±0.005	0.205±0.018
10–28	1.03 ±0.020	1.09 ±0.040
15–28	1.65 ±0.015	1.77 ±0.038
30–28	3.47 ±0.120	3.67 ±0.240
Wf–BSA		
0.5–28	0.061±0.003	0.062±0.004
10 –28	1.50 ±0.055	1.55 ±0.031
50 –28	17.6 ±0.55	17.7 ±0.45
175 –28	108.2 ±0.20	110.0 ±0.96

Free drug concentration (C_f) was determined by the use of the ultrafiltration method (Molcut II, Millipore). HPLC conditions were as follows. Stationary phase: ISRP column (A) (3.0 cm × 4.6 mm i.d., for Im), (B) (10 cm × 4.6 mm i.d., for Wf). Mobile phase: potassium phosphate buffer (pH 7.4, I=0.17). Flow rate: 0.3 ml/min. Detection: UV 315 nm (Im), 313 nm (Wf). Column temperature: 37 °C. Injection volume: 4 ml (Im), 10 ml (Wf).

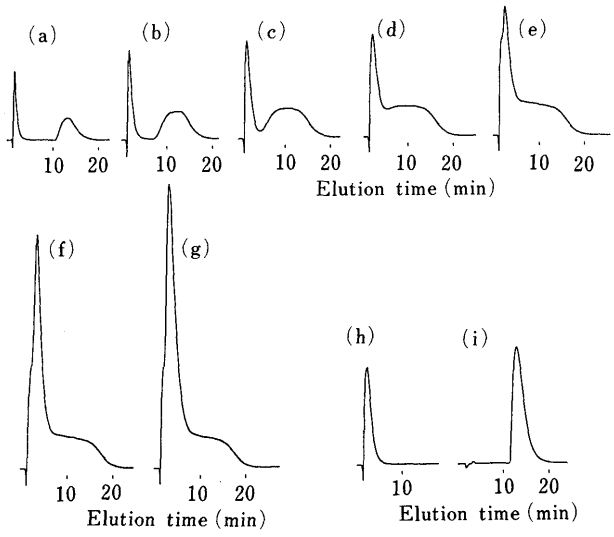

Fig. 3. Effect of Injection Volume on Elution Profile of 28 μM BSA–10 μM Im Mixed Solution

Stationary phase: ISRP column (3 cm × 4.6 mm i.d.). Mobile phase: potassium phosphate buffer (pH 7.4, I=0.17). Flow rate: 0.3 ml/min. Detection: UV 315 nm. Column temperature: 37 °C. Injection volume: 100—900 μl. ((a) 100 μl, (b) 200 μl, (c) 300 μl, (d) 400 μl, (e) 500 μl, (f) 700 μl, (g) 900 μl, (h) 28 μM BSA 400 μl, (i) 10 μM Im 400 μl.)

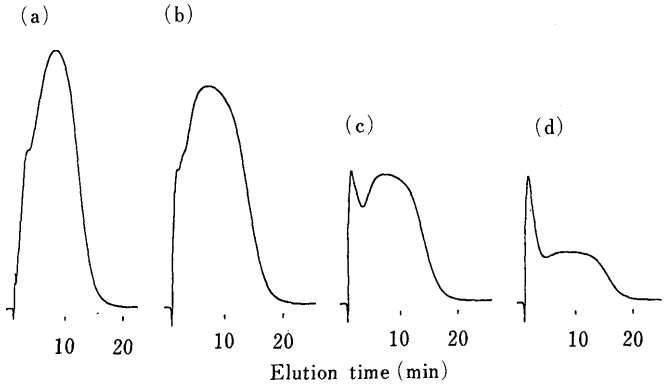

Fig. 4. Elution Profile in Frontal Analysis of 28 μM BSA and 15—100 μM Im Mixed Solution

Stationary phase: ISRP column (3 cm × 4.6 mm i.d.). Mobile phase: potassium phosphate buffer (pH 7.4, I=0.17). Flow rate: 0.3 ml/min. Detection: UV 315 nm. Column temperature: 37 °C. Injection volume: 400 μl. (a) Im 100 μM, (b) Im 50 μM, (c) Im 30 μM, (d) Im 15 μM. (a)—(d) BSA 28 μM.

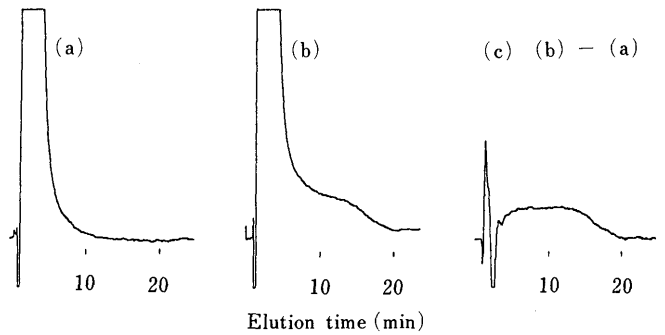

Fig. 5. Frontal Analysis of 28 μM BSA–0.5 μM Im Mixed Solution

Stationary phase: ISRP column (3 cm × 4.6 mm i.d.). Mobile phase: potassium phosphate buffer (pH 7.4, I=0.17). Flow rate: 0.3 ml/min. Detection: UV 315 nm. Column temperature: 37 °C. Injection volume: 400 μl. (a) BSA 28 μM, Im 0 μM; (b) BSA 28 μM, Im 0.5 μM.

injection volume was kept 400 μl. Figure 4 shows the elution profiles of 15—100 μM Im and 28 μM BSA mixed solutions. In all elution profiles, the plateau appeared. These plateau regions did not contain BSA, because BSA was eluted almost completely before this region (see Fig. 3h). As the drug concentration became higher, the plateau range became narrower. The plateau could be observed, although it was very narrow, even in the case of 100 μM Im–28 μM BSA mixed solution (Fig. 4a). This was contrastive with the above mentioned result that when Im concentration was high, it was almost impossible to observe the γ-plateau following the β-plateau after the injection of a larger sample volume. When the Im concentration was higher than 100 μM, an injection of more than 400 μl of the sample solution was necessary to obtain the plateau region. When the drug concentration and, consequently, the plateau height was low, the shape of the plateau region was significantly interfered with by the tailing of the BSA peak. For example, when a 400-μl portion of 0.5 μM Im and 28 μM BSA mixed solution was applied, the plateau region in the elution profile was not flat (Fig. 5b) because of the overlap of the BSA peak tailing (Fig. 5a). However, when the elution profiles of BSA alone (Fig. 5a) and of the mixed solution (Fig. 5b) were measured separately and were memorized by a data analyzer (Chromatopac C-R3A), a flat plateau could be obtained (Fig. 5c) by subtraction of the former chromatogram from the latter.

Table III lists the Im concentration corresponding to the plateau height (C_p). For comparison, the free Im concentrations in these mixed solutions (C_f) were measured by means of the ultrafiltration method. The C_p values correlated well with the C_f values. The linear regression line was $C_p = 0.926 \times C_f + 0.0839$ and the correlation coefficient was 0.9998. These results indicate that the ISRP silica column can determine free drug concentration with good reproducibility and with a relatively small injection volume. It is expected that when a drug is more hydrophobic and is retained to the ISRP silica support more strongly, frontal analysis can be done with a shorter ISRP silica column and, consequently, with a smaller sample volume. Therefore, the present high-performance frontal analysis is particularly advantageous for hydrophobic drugs which are often adsorbed onto the dialysis membrane and ultrafiltration membrane.

Since the free fraction of the drug which binds strongly with plasma proteins is usually less than a few percent, it is of importance to investigate whether the free drug can be determined by high-performance frontal analysis when the volume of free drug percent is at such a low level. Thus, 550 μM BSA–50 μM Im mixed solution was subjected to high-performance frontal analysis. The free Im concentration in this solution determined by use of the ultrafiltration method was 0.361 μM, and the free fraction was very low (0.722%). As shown in Fig. 6, a plateau appeared on injection of 90 μl of the mixed solution onto the 15 cm ISRP silica column. The Im concentration corresponding to this plateau height was 0.388 μM. This result indicates the applicability of this method to samples with low free drug fraction.

Figure 7 shows the elution profiles of 400-μl portions of 10 μM Im–28 μM BSA mixed solution at several flow rates. The effect of flow rate was not great, and the plateau region was observed even at the high flow rate (0.6 and 1.2 ml/min), which is favorable for rapid analysis.

In gel filtration frontal analysis, the durability of the gel is often an important problem, because large injection volumes cause a rapid decrease in the column efficiency. In the present experiment, even after about 100 ml of the drug–BSA mixed solution had been applied in total onto

TABLE III. Concentration of γ-Plateau (C_p) and Free Concentration (C_f) of Im

Sample solution (μM) Im–BSA	C_p (μM) $n=4$	C_f (μM) $n=4$
0.5–28	0.0541 ± 0.00593	0.0510 ± 0.00780
2 –28	0.188 ± 0.0054	0.205 ± 0.0175
10 –28	1.07 ± 0.009	1.09 ± 0.040
15 –28	1.66 ± 0.045	1.77 ± 0.038
30 –28	3.61 ± 0.045	3.67 ± 0.240
50 –28	6.43 ± 0.093	6.67 ± 0.093
75 –28	10.3 ± 0.03	10.9 ± 0.36
100 –28	15.2 ± 0.09	16.5 ± 0.25

Free drug concentration (C_f) was determined by the use of the ultrafiltration method (Molcut II, Millipore). Stationary phase: ISRP column (A) (3.0 cm × 4.6 mm i.d.). Mobile phase: potassium phosphate buffer (pH 7.4, I = 0.17). Flow rate: 0.3 ml/min. Detection: UV 315 nm. Column temp.: 37 °C. Injection volume: 400 μl.

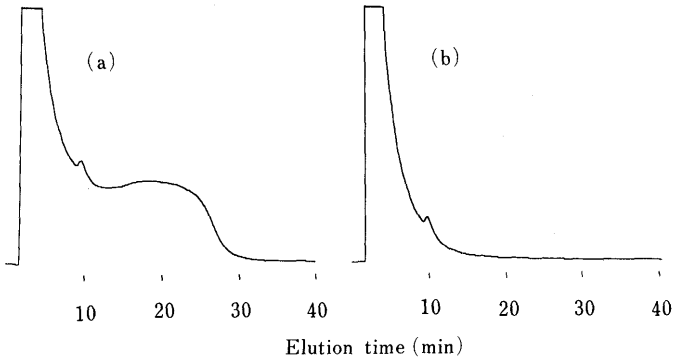

Fig. 6. High-Performance Frontal Analysis of 550 μM BSA–50 μM Im Mixed Solution

Stationary phase: ISRP column (15 cm × 4.6 mm i.d.). Mobile phase: potassium phosphate buffer (pH 7.4, I = 0.17). Flow rate: 0.6 ml/min. Detection: UV 260 nm. Column temperature: 37 °C. Injection volume: 90 μl. (a) BSA 550 μM, Im 50 μM; (b) BSA 550 μM, Im 0 μM.

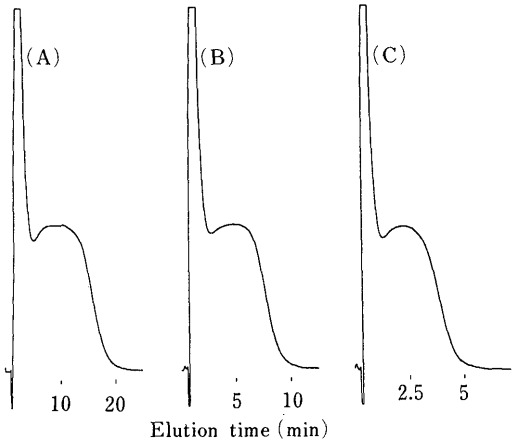

Fig. 7. Effect of Flow Rate on High-Performance Frontal Analysis

Stationary phase: ISRP column (3 cm × 4.6 mm i.d.). Mobile phase: potassium phosphate buffer (pH 7.4, I = 0.17). Flow rate: (A) 0.3, (B) 0.6, (C) 1.2 ml/min. Detection: UV 315 nm. Column temperature: 37 °C. Injection volume: 400 μl.

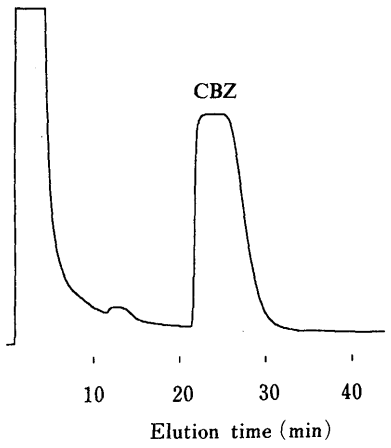

Fig. 8. High-Performance Frontal Analysis of 8 μg/ml Carbamazepine (CBZ) in Human Plasma

Stationary phase: ISRP column (15 cm × 4.6 mm i.d.). Mobile phase: potassium phosphate buffer (pH 7.4, I = 0.17). Flow rate: 1.2 ml/min. Detection: UV 300 nm. Column temperature: 37 °C. Injection volume: 1.8 ml.

the ISRP silica column (A), the plateau region still appeared. This means that in the present method with smaller injection volume, one ISRP silica column (A) can be used more than 250 times for analyses of the Im–BSA mixed solution. Sometimes the column pressure increased because of the accumulation of BSA on the stainless steel inlet frit, but it could be cleaned up by ultrasonification in methanol.

To demonstrate the applicability of this high-performance frontal analysis to body fluid, human plasma containing CBZ was analyzed. The therapeutic range of CBZ concentration is very narrow (4—10 μg/ml), and hence monitoring of the plasma level is of clinical importance.[3] Figure 8 shows the chromatogram of 8.00 μg/ml CBZ in human plasma. CBZ was separated well from the blank peak, and gave a clear and wide plateau. The CBZ concentration calculated from this plateau height was 1.97 μg/ml, which agreed well with the free CBZ concentration determined by means of the ultrafiltration method (2.08 μg/ml). Furthermore, it is interesting that the CBZ concentration calculated from the area of this plateau was 8.06 μg/ml, which agreed well with the total CBZ concentration of this plasma sample. This implies that both free and total drug concentrations can be determined simultaneously by a single analysis based on the height and area of the drug plateau, respectively. Further investigation of the clinical utility of this method in therapeutic drug monitoring is proceeding.

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