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High-performance frontal analysis for drug–protein binding study☆

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Abstract

High-performance frontal analysis (HPFA) is a novel analytical method which enables simultaneous determination of total and unbound drug concentrations under drug–plasma protein binding condition. HPFA can be achieved using separation systems such as HPLC and CE. This paper deals with the principle and feature of HPFA method and its application to the stereoselective protein binding study. HPFA allows a simple analysis following direct sample injection, and does not suffer from undesirable drug adsorption on membrane nor leakage of bound drug through the membrane which are often encountered in conventional ultrafiltration and dialysis methods. HPFA can be easily incorporated into on-line HPLC system. By coupling HPFA with a chiral HPLC column, the unbound concentration of a racemic drug can be determined enantioselectively. The detection limit can be improved by coupling of HPFA with a preconcentration column. High-performance capillary electrophoresis/frontal analysis (HPCE/FA) enables to determine unbound concentrations enantioselectively with ultramicro injection volume, and is hence useful especially for the binding study of proteins which are scarce and difficult to obtain.

Keywords: Albumin; α_1-Acid glycoprotein; Capillary electrophoresis; Chiral separation; Frontal analysis; High performance liquid chromatography; Protein binding

1. Introduction

A drug in plasma binds, more or less, to plasma proteins such as albumin and α_1-acid glycoprotein, and quickly establishes binding equilibrium [1–3]. Unbound drugs in plasma can easily reach the target organ, whereas bound drugs are hard to pass through the blood capillary wall. Consequently, plasma protein binding has a significant effect on the pharmacokinetic and pharmacodynamic properties of a drug. Unbound drug concentrations show better correlation to the pharmacological activity than the total drug concentration. Also, pharmacokinetic properties such as hepatic metabolism rate, renal excretion rate, biomembrane partition rate and steady-state distribution volume are a function of unbound drug

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fraction (unbound/bound concentration ratio). Therefore, quantitative investigation on protein binding is essential to pharmacokinetic study and therapeutic dosing regimen.

Almost half of all chiral drugs are clinically used as racemates, whereas it is often the case that either one of the enantiomers shows pharmaceutical activity. The protein binding property of a racemic drug is potentially different between enantiomers, which often causes the difference in their pharmacokinetic character [4,5]. A stereoselective protein binding study is, hence, essential for the development of new racemic drugs and for the safety in their clinical use.

Protein binding of a drug is reversible and kinetically rapid interaction. Therefore, it should be analyzed without disturbing binding equilibrium. So far, equilibrium dialysis and ultrafiltration followed by HPLC assay have been commonly used to determine unbound drug concentrations. However, these conventional methods involve problems such as drug adsorption onto the membrane and leakage of bound drug through the membrane. In addition, drug–protein binding equilibrium may deviate by the change in the sample concentration during filtration. Therefore, it is recommended to collect the filtrate less than one-fifth of the total sample volume [6]. These problems become serious especially in the case of a highly protein-bound drug. For example, when 99% of the total drug is bound, only a 1% leakage of the protein-bound drug through the filter membrane causes approximately 2-fold overestimation of the unbound drug concentration, and only 0.2% of the total drug amount can be subjected to the analysis. To overcome these difficulties, we developed a novel chromatographic method, named high-performance frontal analysis (HPFA) [7–23]. In this paper, the principle of HPFA and its applicability to the stereoselective protein binding study are reviewed.

2. Principle of high performance frontal analysis

HPFA uses a restricted-access type HPLC column [24,25]. This type of columns have the nature to exclude large molecules of plasma protein but to retain a drug of small molecular size, which allows a direct injection analysis of plasma samples without deproteinization. Originally, these type of columns were developed for the determination of total (bound + unbound) drug concentration. When a small volume of drug–protein mixed solution is directly injected on to this type of HPLC column, the sample solution is diluted with mobile phase, to result in complete and rapid release of bound drug. Thus, total drug (bound + unbound) is retained on the stationary phase ligands, and eluted out of the column to give a single sharp peak. Addition of an organic modifier into the mobile phase serves for the quicker release of the bound drug and improves the peak shape. The sample injection volume should be small enough to avoid peak deterioration due to protein binding [26]. Although total drug concentration can be calculated from the peak area or peak height, no information of unbound drug concentration can be obtained.

On the other hand, HPFA requires an excess volume of sample injection and a mild mobile

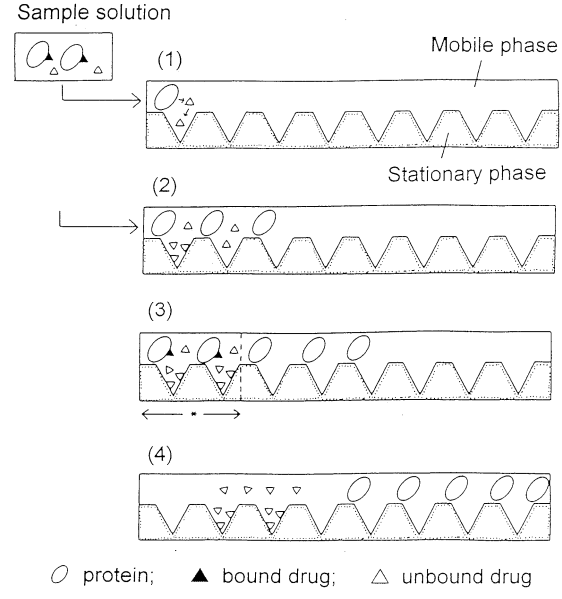

Fig. 1. Schematic view of high performance frontal analysis [8].

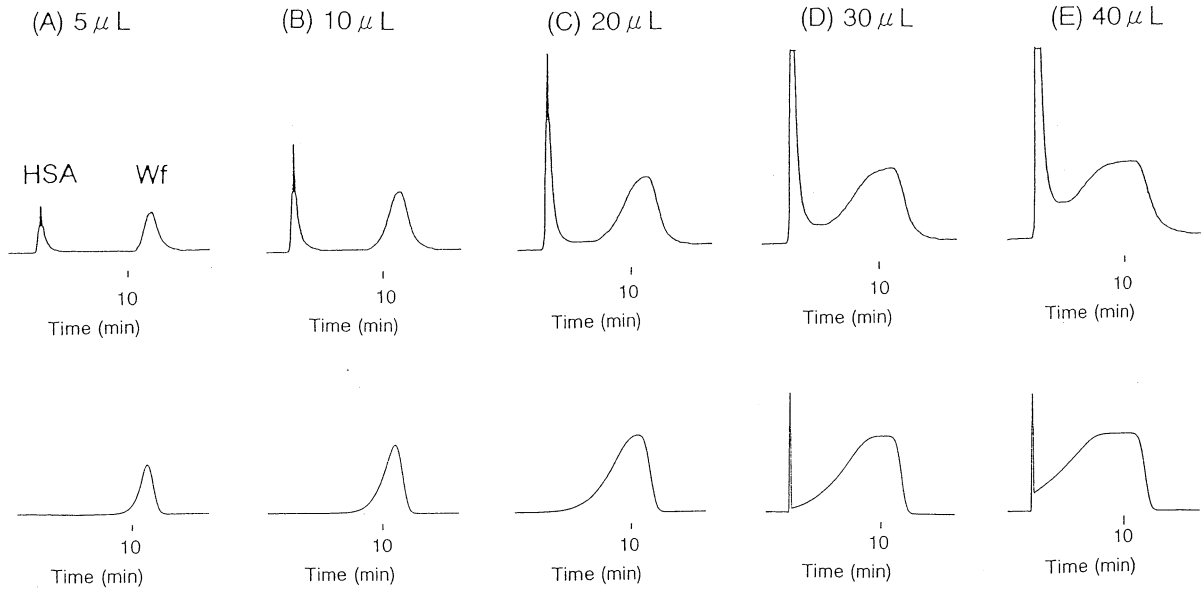

Fig. 2. Effect of injection volume on elution profile of 200 μM Wf and 550 μM HSA mixed solution [18]. Column; Pinkerton column (15 cm × 4.6 mm, i.d.). Mobile phase, phosphate buffer (pH 7.4, $I = 0.17$). Flow rate, 0.5 ml min^{-1}. Detection, UV 308 nm. Temperature, 37°C. Injection volume, 5–40 μl. Binding parameters, $K = 1.96 \times 10^5$ M^{-1}, $n = 1.24$.

phase condition (usually physiological pH 7.4 of phosphate buffer without adding any organic modifier) so as not to disturb the binding equilibrium. Fig. 1 shows the schematic view of HPFA method. When we inject an increased volume of sample solution directly on to the HPFA column, the release of bound drug from protein is apparently suppressed, and finally an equilibrium zone is generated near the top of the column (see Fig. 1(3)). In this zone, two different equilibrium states can be established simultaneously. One is the chromatographic partition equilibrium inside the micropores, and the other is drug–protein binding equilibrium in the interstices (outside the micropores). The drug concentration in the stagnant flow of mobile phase in the micropores is equal to the unbound drug concentration in the bulk mobile phase in the interstices. Since the protein concentration is the same with that in the initial sample solution, the unbound drug concentration in the mobile phase is also equal to that in the initial sample solution. That is, the mobile phase in the interstices is replaced by the sample solution.

The binding equilibrium is kept constant during the separation between drug and protein, because plasma protein binding is a reversible and kinetically rapid process. Then, the unbound drug zone is generated in the column (Fig. 1(4)). This drug zone is eluted as a trapezoidal peak with a plateau region, and the drug concentration in the plateau region is equal to the unbound drug concentration in the initial sample solution. The plateau height and the peak area correspond to the unbound drug concentration and total drug concentration, respectively. This is the principle of the HPFA method, and the unbound drug concentration is determined from the plateau height or by on-line or off-line analysis of the plateau region. This consideration is verified by theoretical and experimental studies [18].

Fig. 2 compares the chromatograms of warfarin (Wf)–human serum albumin (HSA) mixed solution (upper) and the calculated elution profiles based on plate theory (lower). Phosphate buffer ($I = 0.17$) of physiological pH (7.4) was used as the mobile phase and the sample solvent. As the injection volume increased, the Wf peak broad-

ened toward the protein peak. When 30 or 40 μl portion of sample solution was applied, the Wf peak reached maximum height. Further increases in the injection volume did not raise the plateau height. The observed drug elution profile can be well simulated, and the unbound drug concentration in the plateau concentration agrees with the theoretical unbound concentration. This result theoretically confirms the principle of HPFA method (REF). The reliability of HPFA was further confirmed by comparison with a conventional ultrafiltration method using several drugs with different natures such as indometacin, warfarin, salicylate, acetazolamide, carbamazepine, ketoprofen and diclofenac. In every case, good agreement was observed between both methods, though the sample volume is usually far smaller than the conventional methods.

Since appearance of plateau region is essential in HPFA, the injection volume should be large enough to obtain a plateau region. It was found theoretically [18] and experimentally [10] that the minimum injection volume (MIV) necessary for HPFA depends on several factors. MIV increases with increase in unbound drug fraction,and the capacity factor of drug or column length. MIV decreases with increase in binding affinity or the theoretical plate number of the HPFA column.

3. Features of high performance frontal analysis

3.1. Simultaneous determination of total and unbound drug concentration

When a drug peak is separated from protein peak, HPFA allows simultaneous determination of total and unbound drug concentrations by a single analytical run [8]. Fig. 3 shows the HPFA profile of carbamazepine in human plasma. The trapezoidal drug peak was well separated from the first eluted large peak due to plasma proteins. Then, we can determine not only the unbound drug concentration from the plateau height, but also the total drug concentration from the peak area, as shown in Table 1 [8].

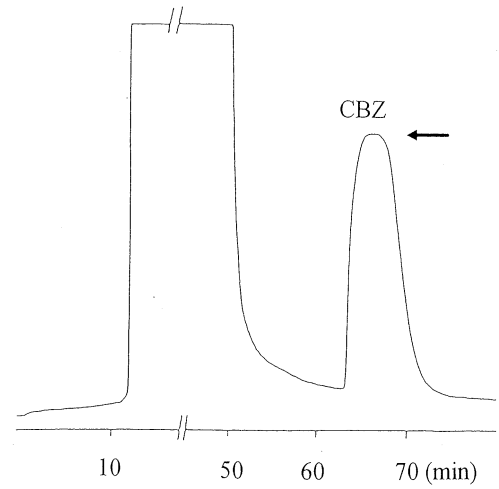

Fig. 3. HPFA profile of human plasma sample containing 4 μg ml^{-1} carbamazepine (CBZ) [8]. Column; Pinkerton column (15 cm × 4.6 mm i.d.). Mobile phase, phosphate buffer (pH 7.4, $I = 0.17$). Flow rate, 0.1 ml min^{-1} (0–50 min), 1.2 ml min^{-1} (after 50 min). Injection volume, 1.9 ml. Detection, UV 300 nm. Column temperature, 37°C.

3.2. Regulation effect

Fig. 4 is a schematic comparison between the HPFA and ultrafiltration method. In the ultrafiltration method, the unbound drug is separated

Table 1
Total and unbound CBZ concentrations in human plasma determined by high performance frontal analysis[a]

CBZ added in plasma	HPFA		Ultrafiltration
	Total CBZ conc. (μg ml^{-1})	Unbound CBZ conc. (μg ml^{-1})	Unbound CBZ conc. (μg ml^{-1})
4	3.93 ± 0.037	1.1 ± 0.010	1.15 ± 0.0270
12	11.9 ± 0.098	3.32 ± 0.054	3.64 ± 0.0571

[a] Mean ± SD ($n = 5$).

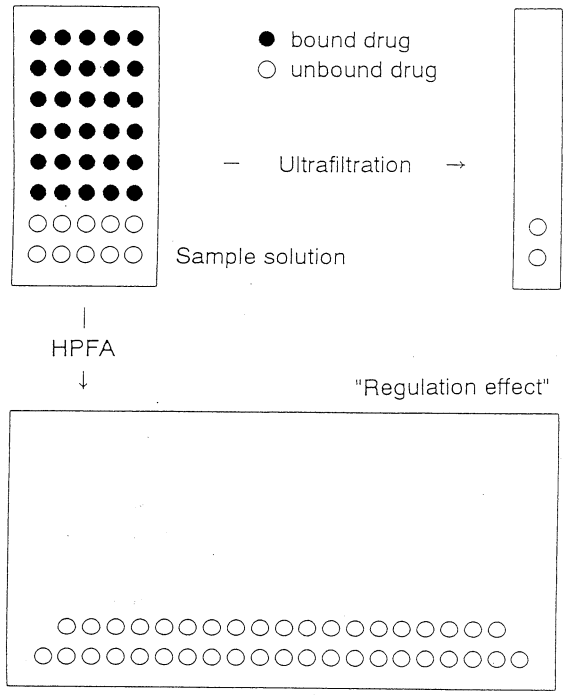

Fig. 4. Schematic comparison between high performance frontal analysis and ultrafiltration method [14].

from the bound drug by using the membrane. Since the volume of the filtrate should be kept less than one-fifth of the sample volume in order to minimize the deviation of binding condition, a very small amount of the unbound drug can be subjected to the subsequent HPLC assay. On the contrary, in HPFA, the bound drug is not separated from the unbound drug, but is converted into the unbound form to give a single zonal peak. Since the amount of drug injected into the column is naturally the same with the amount of drug eluted out of the column, the elution volume of the plateau region (plateau volume) becomes much larger than the injected volume. Therefore, we can subject the unbound drug to subsequent HPLC assay on a much larger scale than (but the unbound drug concentration itself is equal to) that in the case of ultrafiltration method [14].

This effect, named the 'regulation effect', is prominent especially in cases of strong protein binding. The sample solution with higher bound drug fraction gives the larger plateau volume. Therefore, the detectability of low level unbound drug concentration can be dramatically improved by pre-concentrating the unbound drug in a large volume of plateau region [14,16,17,20]. For example, 0.274 ± 0.026 nM ($n = 5$) of unbound troglitazone in human plasma (bound fraction, 99.89%) was determined with good reproducibility by using common UV detection (at 230 nm) [17].

3.3. On-line high performance frontal analysis–chiral-HPLC system for enantioselective protein binding study

In the HPFA of a racemic drug, the plateau region contains unbound forms of both enantiomers, and the unbound concentration of each enantiomer can not be determined separately from the plateau height. However, if HPFA is coupled with a chiral HPLC system, the unbound drug concentration can be determined enantioselectively.

Fig. 5 shows a typical on-line HPFA–chiral HPLC system. The HPFA, extraction and chiral separation columns are connected in series via two switching valves. When a chiral drug–protein mixed solution is injected on to the HPFA column, we obtain a plateau region containing a mixture of unbound drug enantiomers. By switching the four-port valve, a given volume of the plateau region is transferred (heart-cut) into the extraction column. The unbound drug is completely trapped on the extraction column. Then, the unbound drug is transferred on to the chiral HPLC column by the switching of the six-port valve for enantioseparation. The peak area of each enantiomer gives the amount of the trapped enantiomer. By dividing the amount by the heart-cut volume, we can calculate the unbound drug concentration enantioselectively. So far, protein bindings of several racemic drugs, such as warfarin [9,19], fenoprofen [13], ketoprofen [11], nilvadipine [14] and BOF-4272 [16], have been analyzed enantioselectively using an on-line HPFA–chiral HPLC coupling system.

BOF-4272 (Sodium (*R*,*S*)-8-(3-methoxy-4-phenylsulfinyl-phenyl)-pyrazolo [1,5-a]-1,3,5-triazine-4-olate monohydrate) is a newly synthesized

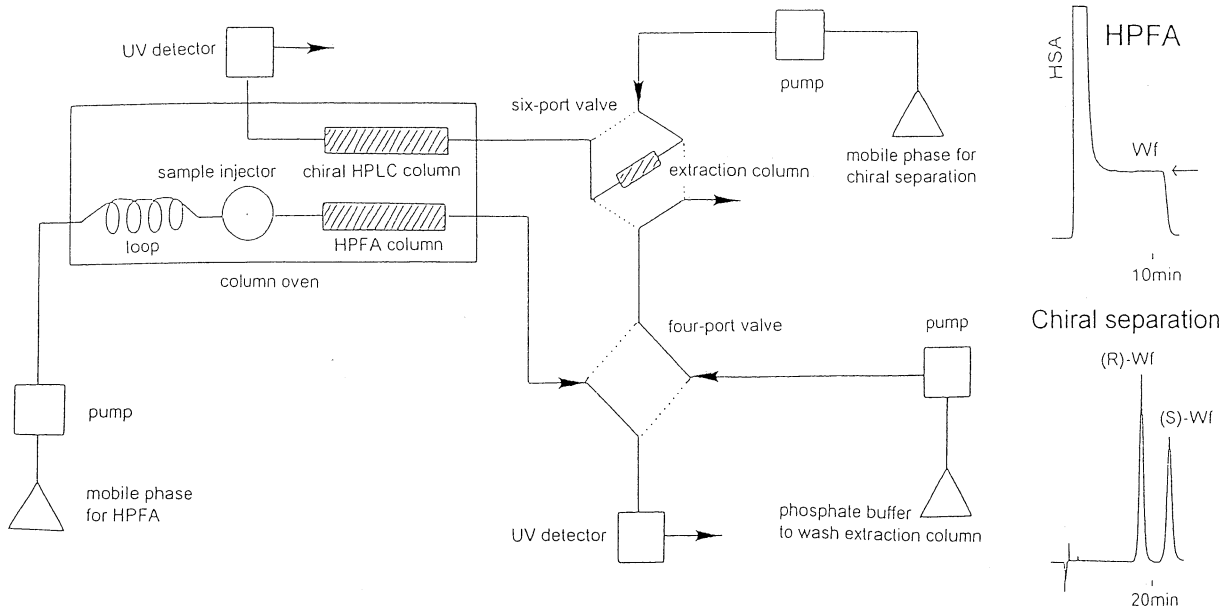

Fig. 5. Schematic diagram of the on-line high performance frontal analysis–chiral HPLC system [16].

xanthine oxidase inhibitor under developing for treatment of hyperuricemia and gout [27]. BOF-4272 has a chiral center at the sulfur atom, and the (*S*)-isomer shows the pharmaceutical activity. Accurate and precise measurement of the unbound concentrations is impossible by any conventional methods because of the low level of unbound concentration due to strong plasma protein binding and the severe adsorption onto the membrane. HPFA is the only method applicable to the binding assay of this drug. Table 2 shows the unbound concentrations of BOF-4272 enantiomers in HSA solution [16]. Interestingly, the enantioselectivity in BOF-4272–HSA binding changes depending on the total drug concentration. In the case of the low total drug concentration, the unbound concentration of the (*R*)-isomer was significantly larger ($P < 0.01$) than that of the (*S*)-isomer, while reverse is true in the case of higher concentrations. To elucidate the inversion of enantioselectivity, the binding parameters were estimated by Scatchard analyses. The binding constant (K) of the (*S*)-isomer is $2.32 \times 10^5\ M^{-1}$, which is almost twice larger than that of the (*R*)-isomer ($1.22 \times 10^5\ M^{-1}$). On the other hand, the number of binding sites per protein molecule (n) of the (*R*)-isomer is 2.30, which is almost twice that of the (*S*)-isomer (1.30). Based on these binding data, the concentration-dependent enantioselectivity can be delineated as follows. When the total drug concentration is much lower than the protein concentration, plenty of binding sites remain unoccupied. In such a case, the difference in the binding constant contributes to the enantioselective protein binding much more prominently than that in the 'n' value. As a result, the (*S*)-isomer with the larger K shows lower unbound concentration than the antipode. On the contrary, when

Table 2
Unbound concentrations of BOF-4272 enantiomers in 550 μM HAS (pH 7.4, $I = 0.17$, 37°C) determined by high performance frontal analysis[a]

Total drug concentration (μM)	Cu(*R*) (nM)	Cu(*S*) (nM)	*R*/*S*
0.5	1.42 ± 0.025	1.23 ± 0.018	1.15
400	1560 ± 91	2090 ± 182	0.748

[a] Mean ± SD ($n = 5$). Cu(*R*) and Cu(*S*) represent the unbound concentration of (*R*)-isomer and (*S*)-isomer, respectively.

the drug concentration is high, and a large number of binding sites are occupied, the number of vacant binding site becomes crucial. As a result, the unbound concentration of the (*R*)-isomer having the larger '*n*' value becomes lower than the (*S*)-isomer. With further increase in the total drug concentration, the unbound fractions of both enantiomers become near unity, and consequently the enantioselectivity will diminish.

4. High-performance frontal analysis/capillary electrophoresis for ultramicro binding study

Plasma protein binding in vivo is in the state of variable and complicated binding equilibrium. In case of a hydrophobic basic drug, albumin, AGP and lipoproteins often contribute to the plasma protein binding simultaneously. The plasma concentration of AGP as well as its microheterogeneity may change depending on disease state [28]. Plasma lipoproteins are classified into several subclasses depending on density such as high-density lipoprotein (HDL) and low-density lipoprotein (LDL). Individual variation and difference between the sexes and state of disease are also observed in their plasma concentrations. These variations will cause the change in the protein binding condition. The binding study of each protein species is important for the entire understanding of plasma protein binding. For this purpose, it is desirable to develop an analytical method which allows binding assays with small sample volumes. We developed high-performance capillary electrophoresis/frontal analysis (HPCE/FA) and applied it to protein binding study of basic drugs [21–23].

In HPCE/FA, both hydrodynamic injection and electrokinetic injection are available. In HPCE/FA following hydrodynamic injection, frontal analysis occurs inside the capillary to generate the unbound drug zone. In HPCE/FA with electrokinetic injection, the principle of frontal analysis effects the selective introduction of the unbound drug on to the capillary column.

In HPCE/FA following hydrodynamic injection, a plug of drug–protein mixed solution (a few hundred nanolitres) is introduced hydrodynamically on to the capillary column filled with a neutral run buffer, and positive voltage is applied on the sample injection side. In the neutral solution (pH 7.4), basic drugs such as propranolol (PRO) and verapamil (VER) are positively charged, while plasma proteins such as HSA and AGP have a net negative charge. The unbound drug migrates much faster than the protein and the bound drug. While the unbound drug is separated from protein, their binding equilibrium is kept constant based on the principle of frontal analysis. Finally, the whole drug migrates as the unbound drug zone [21]. This zone is then separated into two zones of enantiomers by the chiral selector dissolved in the run buffer. The unbound concentration of each enantiomer can be calculated from their respective plateau heights.

In HPCE/FA with electrokinetic injection, the electro-osmotic flow is suppressed by coating the column's inner-surface with a neutral polymer such as linear polyacrylamide or by using an acidic run buffer. By applying positive voltage at the sample injection side, the zone of positively charged unbound drug is introduced on to the capillary, while the negatively charged protein and bound drug are not introduced. The decrease in the amount of unbound drug around the capillary end due to injection on to the capillary column can be supplied by the electrophoretic migration of unbound drug from the bulk sample solution. Thus, the principle of frontal analysis effects the selective introduction of the unbound drug on to the capillary. In addition, the sample diffusion contributes to maintain the binding equilibrium around the capillary injection end. After starting electrophoresis, the unbound drug zone migrates through the capillary toward the cathodic end and was detected as a trapezoidal peak with a plateau zone. The unbound drug concentration is then determined from the plateau height. In the case of a chiral drug, the unbound drug zone can be separated into two zones of the enantiomers by the addition of a chiral selector such as cyclodextrin into the run buffer, and the unbound concentration of each enantiomer can be determined from their respective plateau heights. Fig. 6 shows the typical electropherograms of HPCE/FA with electrokinetic injection. The left electropherogram

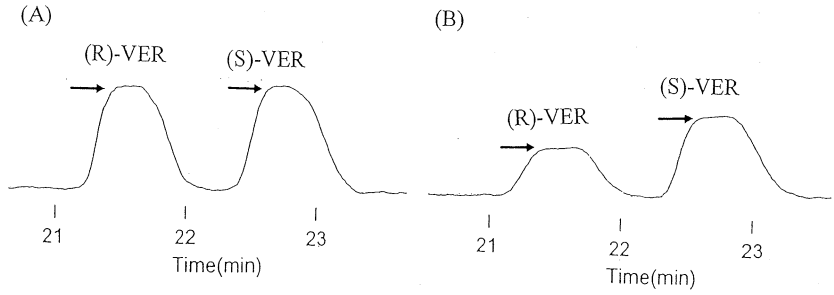

Fig. 6. Electropherograms of; (A) 200 μM racemic VER solution; and (B) 200 μM racemic VER in 550 μM HSA solution obtained by chiral HPCE/FA with electrokinetic injection [22]. Run buffer, 50 mM phosphate buffer (pH 2.5) containing 40 mM trimethyl-β-CD. Capillary, 63 cm (effective length 50 cm), 75 μm i.d. Applied voltage, +18 kV. Detection UV 200 nm.

is due to racemic VER without protein, and the right to racemic VER and HSA mixed solution. VER enantiomers were completely separated by the chiral selector (trimethyl-β-cyclodextrin). The plateau heights in Fig. 6A represent the total drug concentrations of both enantiomers and are equal to each other. The plateau heights in Fig. 6B represent their unbound concentrations. Due to protein binding, their plateau heights became lower than those in Fig. 6A. In addition, the plateau heights are different between the enantiomers, indicating enantioselective protein binding.

Both injection methods gave the same results with good reproducibility, and the results agreed well with those obtained by the conventional ultrafiltration method followed by chiral HPLC analysis. The sample injection volume by the hydrodynamic injection method was about 100–200 nl. This is smaller by more than two orders of magnitude than the sample volume required by the ultrafiltration method (100 μl). The injection amount by the electrokinetic injection method was estimated, judging from the peak area, to be almost the same as that by the hydrodynamic injection method.

AGP is the most important plasma protein responsible for plasma protein binding of a basic drug. AGP molecule contains five *N*-glycan chains which have di-, tri- and tetra-antennary structures, with sialic acids as the terminal group. The glycan structures show microheterogeneity under physiological conditions, and the partially desialylated AGP is known to exist in plasma of patients with liver disease [29,30]. Because sialic acid has a negative charge, it may contribute to the binding of basic drugs with AGP. Therefore, we investigated the function of sialic acid groups at the terminal of AGP glycan chains with respect to chiral discrimination between optical isomers of basic drugs (PRO and VER) using the HPCE/FA method [23]. It was found that the unbound concentration of (*S*)-VER was 1.3 times higher than that of (*R*)-VER in native AGP solution, and this selectivity was not affected by desialylation of AGP. Further, enzymatic elimination of end-terminal galactose residues of the desialylated AGP did not change the binding of either isomer of VER. On the other hand, the unbound concentration of (*R*)-PRO was 1.27 times higher than that of (*S*)-PRO in native AGP solution. Desialylation did not change the unbound concentration of (*R*)-PRO, but caused the unbound concentration of (*S*)-PRO to rise upto the same level of (*R*)-PRO, resulting in the loss of enantioselectivity. This result suggests that the sialic acid residues may be regarded as one origin of enantioselectivity in AGP–PRO binding, while they are not responsible for the enantioselective AGP–VER binding.

5. Conclusion

HPFA has several advantages over conventional methods:(1) direct injection analysis; (2) no

troubles such as leakage of bound drug through membrane and adsorption of drug to the membrane which often cause serious problems in the conventional methods; (3) simultaneous determination of total and unbound drug concentrations; (4) easy to incorporate into an on-line HPLC system and; (5) a 'regulation effect' which allows the determination of a few nanomolar or unbound drug concentration with a relatively small (a few hundred microlitres) sample injection volume.

HPFA is useful especially for the plasma protein binding study of hydrophobic drugs. HPFA is applicable to any binding study provided that the binding is rapid and reversible, and that the difference in the elution time between the interacting substances is sufficient. Further application of HPFA, such as the binding study between endogenous active substances and several kinds of biopolymers, is expected. In addition, HPFA incorporated in capillary electrophoresis allows protein binding analysis with ultramicro sample volume (≈ 100 nl). This method is useful especially for the binding study of proteins which are scarce and difficult to obtain, like desialized glycoprotein [23] and lipoproteins.

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