High-performance capillary electrophoresis/ frontal analysis for stereoselective drug-protein binding study

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High-performance capillary electrophoresis/frontal analysis (HPCE/FA), which enables to determine unbound concentrations enantioselectively with ultramicro injection volume, is a useful method for plasma protein binding study of a chiral drug.

A drug in plasma binds, much or less, to plasma proteins, such as albumin and α_1-acid glycoprotein (AGP), and quickly establishes binding equilibrium. Unbound drug in plasma can easily reach the target organ, whereas bound drug is hard to pass through the blood capillary wall. Therefore, unbound drug concentration shows better correlation to the pharmacological activity than the total drug concentration. Several pharmacokinetic properties such as hepatic metabolism rate, renal excretion rate, biomembrane partition rate and steady-state distribution volume are the function of unbound drug fraction (unbound/total concentration ratio). In addition, protein binding property of a racemic drug is potentially different between the enantiomers, which often causes the difference in their pharmacokinetic character. Stereoselective protein binding study is, hence, essential for the effective development of new racemic drugs and for the safety in their clinical use [1,2]. Recently, high-performance capillary electrophoresis (HPCE) has been applied to drug – plasma protein binding study [3]. This paper briefly reviews our recent study of chiral HPCE/FA method.

Principle of chiral HPCE/FA method

Chiral separation is achieved by adding a suitable chiral selector, such as cyclodextrin, into HPCE run buffer. Since the mobility of the diastereomeric complex between a chiral drug and cyclodextrin is usually lower than that of the free drug, the enantiomer which makes the stronger complex with the chiral selector migrates slower than the antipode, resulting in the chiral separation. We coupled this chiral HPCE with frontal analysis to achieve enantioselective binding analysis [4]. Both hydrodynamic injection and electrokinetic injection are available. In HPCE/FA following hydrodynamic injection, frontal analysis can be performed 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 into capillary.

In HPCE/FA following hydrodynamic injection, a plug of drug-protein mixed solution (a few hundred nanolitter) is introduced hydrodynamically into the capillary filled with a neutral run buffer, and positive voltage is applied to the sample injection side. In the neutral solution (pH 7.4), basic drug such as propranolol (PRO) and verapamil (VER) is positively charged, while human plasma protein such as HSA and AGP have negative net charge. The unbound drug migrates much faster than the proteins and the bound drug. While the unbound drug is separated from protein, their binding equilibrium is kept unchanged based on the principle of frontal analysis. Finally, the whole drug migrates as the unbound drug zone [5], and this zone is then separated into two zones of enantiomers by the chiral selector. The unbound concentration of each enantiomer can be calculated from the plateau height of the respective peaks.

Figure 1 illustrates chiral HPCE/FA with electrokinetic injection. The electroosmotic flow is suppressed by using an acidic run buffer or by coating the inner-surface with a neutral polymer such as linear polyacrylamide. By applying positive voltage to the sample injection side, the zone of positively charged unbound drug is introduced into

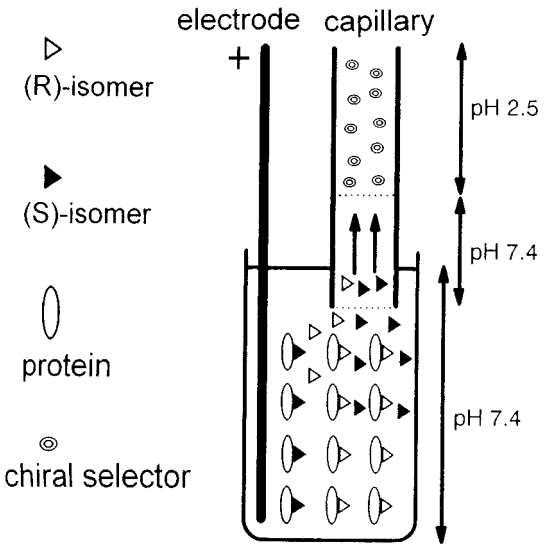

Figure 1. Schematic diagram of chiral HPCE/FA with electrokinetic injection [4].

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capillary, while negatively charged protein and bound drug are not introduced. Since there is no difference in mobility between unbound enantiomers, they are introduced keeping the same *R/S* ratio as that in the sample solution. The decrease in the amount of unbound drug around the capillary end due to injection into capillary can be supported by the electrophoretic migration of unbound drug from the bulk sample and/or by the sample diffusion. Electrophoresis starts after the anodic end of the capillary is immersed into the run buffer. The introduced unbound drug zone is separated by the chiral selector, and two peaks each having a plateau zone are detected. The unbound concentration of each enantiomer can be determined from the plateau height.

In a protein binding study, it is important to investigate the bindability under physiological condition. Therefore, phosphate buffer of physiological pH (=7.4) is used to prepare sample solutions. In case an acidic buffer is used, the protein binding condition in sample solution may be disturbed by the direct contact with the acidic run buffer. To avoid this trouble, a small volume of neutral buffer (pH 7.4) should be introduced prior to the sample introduction.

Figure 2 shows the typical electropherograms of HPCE/FA with electrokinetic injection. The left electropherogram is due to racemic VER solution without protein, and the right is due to the drug and HSA mixed solution. VER enantiomers were completely separated by the chiral selector (trimethyl-β-cyclodextrin). The plateau heights in figure 2A represent the total drug concentrations of both enantiomers and are equal to each other. The plateau heights in figure 2B represent their unbound concentrations. Due to protein binding, their plateau heights are lower than those in figure 2A. In addition, the plateau heights are different between the enantiomers, indicating the enantioselective protein binding.

Both HPCE/FA 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, which demonstrates the reliability of the present chiral HPCE/FA method. The unbound concentration of (*S*)–VER is 1.7 times higher than the antipode, which agrees with the reported result that (*R*)–VER is bound with HSA more tightly than (*S*)–VER [6].

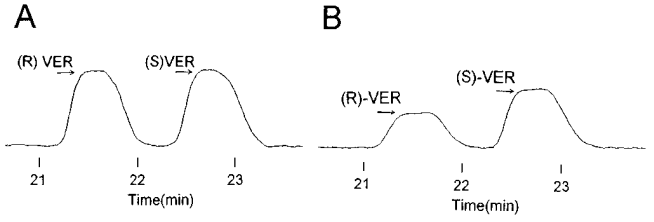

Figure 2. Electropherograms of A) 200 μM racemic VER solution and B) 200 μM racemic VER in 550 μM HSA solution obtained by chiral HPCE/FA with electrokinetic injection [4]. Run buffer, 50 mM phosphate buffer (pH 2.5) containing 40 mM trimethyl-β-CD. Capillary, 63 cm (effective length 50 cm), 75 mm i.d. Applied voltage, +18 kV. Detection UV 200 nm.

The sample injection volume in the hydrodynamic injection was 200 nL. This is smaller by more than two order of magnitude than the sample volume in the ultrafiltration method (100 μL). From the peak area, the sample amount introduced by the electrokinetic injection was estimated to be the same with that by the hydrodynamic injection.

Effect of sialic acid residues of AGP

AGP plays an important role in plasma protein binding of a basic drug. AGP molecule contains five N-glycan chains which have di-, tri- and tetraantennary 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 [7,8]. Because sialic acid has a negative charge, it may contribute to the binding of basic drugs with AGP. Therefore, we investigated the effect of sialic acid residues using the chiral HPCE/FA method. PRO and VER were used as model basic drugs, and their unbound concentrations in native AGP and in enzymatically desialylated AGP solution were compared [9].

In 30 μM racemic VER –40 μM native AGP mixed solution, the unbound concentration of (*S*)–VER (7.35 ± 0.02 μM) is higher than (*R*)–VER (4.75 ± 0.02 μM), and the desialylation gave no change in the unbound concentrations of both enantiomers, which suggests that sialic acid residues do not contribute to chiral recognition in VER-AGP binding.

In 30 μM (*R*)- or (*S*)–PRO and 40 μM native AGP mixed solution, the unbound concentration of (*R*)–PRO (15.0 ± 1.21 μM) was higher than (*S*)–PRO (11.3 ± 0.59 μM). By desialylation, the unbound concentration of (*S*)–PRO increased to 14.4 ± 1.63 μM, while that of (*R*)–PRO did not change (15.0 ± 0.42 μM), resulting in loss of enantioselectivity. This suggests that sialic acid residue(s) are involved in chiral recognition in binding with (*S*)–PRO but not in the binding with (*R*)–PRO. Thus, sialic acid residue(s) could be regarded as the origin of enantioselectivity.

Conclusion

Chiral HPCE/FA allows a simple and quantitative assay of enantioselectivity of plasma protein binding of basic drugs with a ultramicro sample injection volume. It is suggested that sialic acid residues influence the enantioselective binding of chiral basic drugs with AGP in different ways. They are not involved in VER-AGP binding. They participate in the binding with (*S*)–PRO but not with (*R*)–PRO. Chiral HPCE/FA method is useful especially for the binding study of proteins which are scarce and difficult to obtain, like desialylated glycoprotein and lipoproteins.

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