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Frontal analysis of drug–plasma lipoprotein binding using capillary electrophoresis

Takanori Ohnishi [a], Niveen Abdel Latif Mohamed [a], Akimasa Shibukawa [a,*], Yukihiro Kuroda [a], Terumichi Nakagawa [a], Samia El Gizawy [b], Hassan F. Askal [b], Micheal E. El Kommos [b]

[a] *Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan*
[b] *Department of Pharmaceutical Analytical Chemistry, Faculty of Pharmacy, Asiut University, Asiut, Egypt*

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Dedicated to Professor Dr Gottfried Blaschke on the occasion of his 65th birthday

Abstract

High performance frontal analysis coupled with capillary electrophoresis (HPFA/CE) was applied to the ultramicroanalysis of enantioselective binding of drug to plasma lipoproteins. A small volume (ca. 80 nl) of (*R*)- or (*S*)-propranolol (PRO, 25–150 μM) and human high-density lipoprotein (HDL, 2.63 g/l) or human low-density lipoprotein (LDL, 4.37 g/l) mixed solution, which was in the state of binding equilibrium, was introduced hydrodynamically into a non-coated fused silica capillary. Positively charged unbound PRO enantiomers migrated toward cathodic end much faster than negatively charged lipoproteins and the bound form. Once unbound PRO migrated apart from lipoprotein, the bound PRO was quickly released from the lipoprotein to maintain the binding equilibrium. Thus, PRO migrated as a zone in the capillary, giving a peak with a plateau region, where the concentration is the same as the unbound PRO concentration in the original sample solution. The unbound PRO concentration calculated form the plateau height agreed with that determined by a conventional ultrafiltration method used as a reference method. It was found that the bindings of PRO to HDL and PRO to LDL were not enantioselective, while the total binding affinity of PRO to LDL (4.01×10^5 per M) was 17 times higher than that of PRO–HDL binding (2.38×10^4 per M). © 2002 Published by Elsevier Science B.V.

Keywords: Capillary electrophoresis; Frontal analysis; Protein binding; Lipoprotein; Propranolol; Enantiomer

1. Introduction

The interaction with plasma proteins gives significant effect upon pharmacokinetics and pharmacodynamics of drugs [1–3]. This interaction, called as plasma protein binding, is in the state of

* Corresponding author. Tel.: +81-85-753-4531; fax: +81-75-753-4578.
E-mail address: akimasas@pharm.kyoto-u.ac.jp (A. Shibukawa).

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variable and complicated binding equilibrium. Several plasma proteins possibly contribute to the plasma protein bindings of one drug simultaneously, and the overall plasma protein binding is the sum of each protein binding. Therefore, in addition to in vivo binding study using whole plasma, in vitro binding study using each component of plasma proteins is necessary for the detailed elucidation of plasma distribution of drug. In addition, protein binding of a racemic drug is potentially different between the enantiomers, which may result in the enantioselectivity in disposition property [4,5]. Enantioselective plasma protein binding study is, therefore, inevitable for the effective development of a new racemic drug and for the safety in the clinical use.

Albumin and α_1-acid glycoprotein (AGP) are the major proteins responsible for plasma protein binding of drugs. Besides, plasma lipoproteins are also known to contribute to the plasma protein binding of several basic and neutral hydrophobic drugs [6]. Plasma lipoproteins are classified into some subclasses based on the density, such as high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low density lipoprotein (VLDL) and chylomicron (CM). Among these, HDL and LDL are the most important drug-transporting proteins because of their higher plasma concentrations than others. Lipoprotein is a metastable complex of apoprotein and several lipid components. A considerable inter-individual difference as well as variation due to disease state are observed in the plasma concentrations of lipoproteins, which possibly affect the plasma distribution of drug. Since apoprotein and some lipid components such as free cholesterol, cholesterol ester and several phospholipids have chiral nature, lipoproteins may show enantioselective binding property. However, the binding studies of lipoproteins have been reported not so much as those of albumin and AGP mainly because of difficulty in the long-term preservation. The development of a micro-scale binding analysis is a key issue for the progress in drug–lipoprotein binding study.

We have developed high performance frontal analysis/capillary electrophoresis method (HPFA/CE) [7–13]. HPFA/CE is beneficial to the binding study of scarce proteins, because it allows to determine unbound drug concentration with a very small sample size ($\sim$ 100 nl), which is smaller by two orders or more than that needed in the conventional ultrafiltration and equilibrium dialysis methods. This method is free from the errors due to leakage of bound drug through membrane and/or drug adsorption onto membrane which are often encountered in conventional ultrafiltration and dialysis methods. HPFA/CE has been applied to the enantioselective protein binding analysis of human serum albumin (HSA) [8], AGP glycoforms [9,10], AGP genetic variants [11], and native and oxidized lipoproteins [12,13]. HPCE/FA is also applicable to the screening of interactions between β-adrenoceptor blocking drugs and human serum proteins such as HSA, AGP and lipoproteins [14].

In this paper, the applicability of HPFA/CE to the drug–plasma lipoprotein binding was investigated using propranolol (PRO) as a model basic and racemic drug, and the reliability of this method was estimated by comparing it with a conventional ultrafiltration–HPLC method.

2. Materials and methods

2.1. Materials and apparatus

(*R*)- and (*S*)-enantiomers of PRO hydrochloride were purchased from Sigma (St. Louis, MO). The drug–protein mixed solutions were made up in sodium phosphate buffer (pH 7.4, ionic strength 0.17). The capillary electrophoresis system 270A (Applied Biosystems) equipped with an uncoated fused sillica capillary (50 cm, 75 μm i.d., effective length, 30 cm) was used. PRO was monitored at UV 215 nm. Beckman L7-65 was used for ultracentrifugation.

2.2. Preparation of HDL and LDL by sequential ultracentrifugation

Human HDL and LDL were prepared from plasma of a healthy male volunteer by sequential ultracentrifugation method. Briefly, human plasma, the density of which was adjusted as

1.006 g/ml, was ultracentrifuged for 24 h × 50 000 rpm at 4 °C, and the upper fraction was removed. The density of the remainder was adjusted to 1.063 g/ml, and the following ultracentrifugation (20 h × 38 000 rpm, 4 °C) gave the LDL fraction (upper fraction). The density of the lower fraction was adjusted to 1.21 g/ml, and the ultracentrifugation (48 h × 38 000 rpm, at 4 °C) gave the HDL fraction (upper fraction). In the above procedure, the density was adjusted using NaBr. The HDL fraction (density, 1.063–1.21 g/ml) and LDL fraction (density, 1.006–1.063 g/ml) were further purified by size-exclusion HPLC. The HPLC condition was as follows. Column, HiLoad Superdex 200 pg (60 × 2.6 cm i.d., Pharmacia). Mobile phase, sodium phosphate buffer (pH 7.4, $I = 0.17$). Flow rate, 2.0 ml/min. Column temperature, 4 °C. Detection, UV 254 nm. The purified HDL and LDL fractions were concentrated on the membrane (Cemtriplus-10, Amicon) as follows. A 40 ml portion of the LP fraction was centrifuged at 3600 × *g* for 3 h at 4 °C. The final volume was about 5 ml.

2.3. Determination of lipoprotein concentrations

The concentrations of HDL and LDL were calculated as follows. First, the concentration of apoprotein (U, g/l) was measured by modified Lowry method in which SDS solutions were used to prevent the interference from lipid components [15]. Bovine serum albumin (BSA) (fatty acid-free) was used as the standard protein in preparing the calibration line. The concentration of lipoprotein (g/l) was then calculated assuming the w/w contents of apoprotein in HDL and in LDL are 50 and 21%, respectively [6]. The molar concentration of lipoprotein was then calculated assuming the molecular weight of HDL and LDL are 1.8×10^5 and 2.3×10^6 Da, respectively [6].

2.4. Determination of unbound drug concentrations by HPFA/CE

The sample solutions containing physiological concentration of HDL (14.6 μM) or LDL (1.9 μM) and (*R*)- or (*S*)- enantiomer of PRO (pH 7.4, ionic strength 0.17) were introduced hydrodynamically into the capillary (3 s, ca. 80 nl), and a positive voltage (+4 kV) was applied to start electrophoresis. The temperature was set at 25 °C. Unbound PRO enantiomers migrated toward the cathodic end (detection side) much faster than the negatively charged lipoproteins and the bound drug. Since drug–protein binding, in general, is reversible and kinetically rapid, the drug and protein are separated from each other, while the binding condition is kept constant. After the unbound drug moved out, bound drug was quickly released to maintain the same equilibrium condition. As a result, a trapezoidal peak of unbound drug was obtained, and the unbound drug concentration was measured from the plateau height.

A series of the standard PRO solutions without lipoprotein (10, 25, 50, 100 and 150 μM of (*R*)- or (*S*)-PRO in phosphate buffer, pH7.4, ionic strength 0.17) were used to prepare calibration lines. The calibration lines thus obtained indicated good linearity ($R > 0.999$). After each binding analysis, the capillary was washed with 30 mM SDS and run buffer each for 1 min. It is reported that this washing with SDS solution is effective for reproducible and reliable CE analyses of samples containing proteins [16]. No serious trouble due to adsorption of LPs onto inner capillary wall was observed.

2.5. Electrophoretic mobility of lipoproteins in CZE mode

The electrophoretic mobilities of HDL and LDL in CZE mode were measured using an uncoated fused silica capillary (total length 50 cm, effective length 20 cm, i.d. 75 μm). Run buffer was sodium phosphate solution (pH 7.4, ionic strength 0.17) containing 0, 50 or 150 μM (*S*)-PRO. The applied voltage was +4 kV, and the temperature was 25 °C. The lipoproteins were monitored at UV 254 nm.

2.6. Determination of unbound PRO concentration by ultrafiltration-HPLC method

A disposable ultrafiltration followed by HPLC determination was used as a reference standard

method to determine unbound PRO concentrations. A 500 μl portion of PRO–lipoprotein mixed solution was applied to the ultrafiltration kit (Centrifree MPS-3, Amicon), followed by the centrifugation at 100 × *g* for 1 min to suppress the adsorption of the drug on the filter membrane. After the filtrate was discarded, the kit was further centrifuged at 850 × *g* for 1 min to obtain a 100 μl portion of the filtrate containing the unbound drug. All filtration procedures were performed at 25 °C. A 10 μl portion of the filtrate was subjected to the reversed-phase HPLC to determine the unbound drug concentration. The HPLC condition was as follows. Column, YMC-Pack ODS-AK (15 cm × 4.6 mm i.d., 5 μm, YMC, Japan). Mobile phase, 20 mM NaH_2PO_4: acetonitrile = 6:4 (v:v). Flow rate, 1.2 ml/min. Detection, UV 214 nm. Injection volume, 10 μl. Column temperature, 40 °C. The calibration curves were prepared by injecting 10 μl portion of a series of standard solutions containing 10–120 μM (*R*)- or (*S*)-PRO. The good linearity ($R > 0.999$) was obtained as for both PRO enantiomers.

3. Results and discussion

In the frontal analysis using capillary electrophoresis, if the electrophoretic mobility of drug–protein complex is different from that of free protein, the drug concentration in the plateau zone will become different from the original unbound drug concentration, because the binding equilibrium changes during the electrophoretic separation process [17]. However, this problem is negligible in case the binding does not bring about a considerable change in the protein mobility, as in case of warfarin–albumin binding [18]. In the present study, we could neglect this problem, because the change in the electrophoretic mobility of HDL and LDL caused by the addition of 150 μM PRO (less than 5%) was very slight. That is, the electrophoretic mobilities of HDL and LDL in the absence of PRO in the run buffer were -0.00991 ± 0.00051 and -0.00978 ± 0.00015 cm^2/min V, respectively, those in the presence of 50 μM (*S*)-PRO in the run buffer were -0.00978 ± 0.00017 and -0.00968 ± 0.00037 cm^2/min V, respectively, and those in the presence of 150 μM (*S*)-PRO were -0.00950 ± 0.00019 and -0.00985 ± 0.00025 cm^2/min V, respectively ($n = 3$).

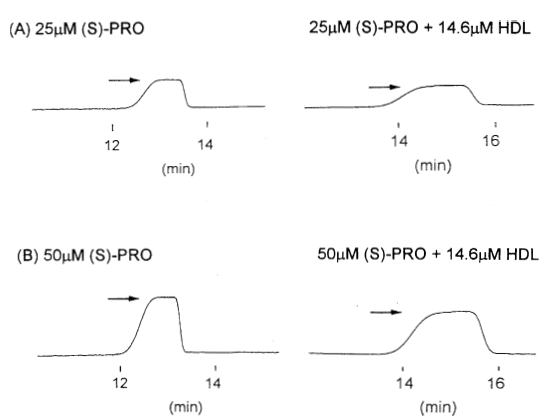

Fig. 1. HPFA/CE profiles of PRO–HDL binding. CE conditions, see text.

Fig. 1 shows HPFA/CE profiles of (*S*)-PRO in HDL solutions. The left side shows the electropherograms of (*S*)-PRO in protein-free sample solutions, where the plateau height represents naturally the total drug concentration. The right side shows the electropherograms of (*S*)-PRO of the same concentration in HDL solutions. Due to protein binding, the plateau height in the right side became lower than that of the protein-free sample solution, and the unbound drug concentration can be determined from this plateau height. Similar electropherograms were obtained from the LDL solutions (figure not shown).

Since lipoprotein is a molecular aggregate of apoprotein and several lipids, two different modes of drug binding would be possible. One is the binding to apolipoprotein, which is site-specific like in case of albumin and AGP. Another is the binding to lipid components, which is non-specific and partition-like. These binding modes can be discriminated by investigating the relation between the unbound drug fraction and the total drug fraction. In case the binding is saturable and unbound drug fraction is increased with increasing total drug concentration, the former binding mode is dominant. On the other hand, in case the binding is non-saturable and the unbound drug

Table 1
Unbound concentration, unbound fraction and R/S ratio of propranolol enantiomers in 14.6 μM human HDL solutions

Total concentration (μM)	(*R*)-PRO		(*S*)-PRO		*R*/*S*
	Unbound concentration (μM)	Unbound (%)	Unbound concentration (μM)	Unbound (%)	
25	18.0 ± 0.13	71.9 ± 0.52	18.1 ± 0.28	72.4 ± 1.21	0.994
50	37.2 ± 0.53	74.5 ± 1.06	37.2 ± 0.17	74.3 ± 0.34	1.00
100	74.2 ± 0.29	74.2 ± 0.29	75.1 ± 0.29	75.1 ± 0.29	0.988
150	114 ± 0.87	76.3 ± 0.58	110 ± 0.85	73.4 ± 0.57	1.04

Mean ± S.D. ($n = 3$).

Table 2
Unbound concentration, unbound fraction and R/S ratio of propranolol enantiomers in 1.90 μM human LDL

Total concentration (μM)	(*R*)-PRO		(*S*)-PRO		*R*/*S*
	Unbound concentration (μM)	Unbound (%)	Unbound concentration (μM)	Unbound (%)	
25	13.8 ± 0.29	55.3 ± 1.16	13.9 ± 0.82	55.5 ± 3.26	0.993
50	28.3 ± 0.77	56.6 ± 1.53	28.1 ± 0.96	56.2 ± 1.92	1.01
100	57.1 ± 1.29	57.1 ± 1.29	56.9 ± 0.82	56.9 ± 0.82	1.00
150	87.2 ± 0.63	58.1 ± 0.42	87.7 ± 0.85	58.5 ± 0.56	0.994

Mean ± S.D. ($n = 3$).

fraction is constant regardless of the total drug concentration, the latter binding mode is dominant. Table 1 shows the unbound concentrations and the unbound fraction of PRO enantiomers in physiological concentration of HDL sample solutions determined by the present method. The total drug concentration ranges from 25 to 150 μM, while the concentration of HDL (14.6 μM) was constant. The R/S ratio of the unbound concentration was almost unity, which means no enantioselectivity in binding to HDL. In addition, the unbound fraction was almost constant (ca. 74%) regardless of the total drug concentration. The major protein constituents in HDL are apolipoprotein A-I and A-II, and one HDL contains five to six molecules of these A apolipoproteins [19]. Therefore, the apolipoprotein concentration in the sample solutions can be estimated as 73–88 μM. In this study, the drug binding was not saturated even when the total drug concentration was 1.7 times higher or more than the apolipoprotein concentration ([PRO] = 150 μM). Table 2 shows the unbound concentrations of PRO enantiomers in the physiological concentration of LDL sample solutions determined by the present method. Similarly to PRO–HDL binding in Table 1, no enantioselectivity was found in PRO–LDL binding, and the unbound fraction is constant regardless of the total drug concentration. Apolipoprotein B-100 is the major protein constituent of LDL. Since one LDL particle contains one apolipoprotein B-100 molecule [20], the apolipoprotein concentration in the present sample solutions is estimated as 1.9 μM. In this study, the drug binding was not saturated even when the total drug concentration was about 80 times higher than the apolipoprotein concentration ([PRO] = 150 μM). These results indicate that the partition-like binding to lipid phase seems to be dominant in PRO–HDL binding and in PRO–LDL binding. This conclusion verifies the previous finding [21]. Usually chiral recognition occurs at specific

binding site such as in case of bindings to albumin and to AGP. However, the dominant drug binding sites of HDL and LDL are lipid components, and the drug bindings of HDL and LDL are non-specific. As a result, no enantioselectivity was found.

Table 3 shows the total binding affinity (nK) between PRO enantiomers and lipoproteins calculated from the data shown in Tables 1 and 2. The nK for PRO–HDL binding is 2.4×10^4 per M, and the nK value for PRO–LDL binding is 4.0×10^5 per M. LDL, containing higher lipid fraction (ca. 80%) than HDL (ca. 50%), has 17 times stronger affinity than HDL, which also supports that the binding to the lipid phase rather than specific binding to apoprotein is dominant in PRO–lipoprotein interaction. These nK values estimated by HPFA/CE are in agreement with those estimated by equilibrium dialysis (4.6×10^4 and 1.8×10^5 per M for PRO–HDL binding and PRO–LDL binding, respectively) [21].

Frontal analysis is applicable, as far as the reversible binding equilibrium is rapidly established like in the case of binding to albumin or AGP. Otherwise, the binding will not reach the equilibrium state, resulting in the lower unbound drug concentration in the plateau region. Unlike these plasma proteins, to which drug is bound stoichiometrically at specific binding site(s), lipoproteins show nonspecific and partition-like binding character. It is, therefore, important to confirm that drug binding to lipoprotein occurs rapidly enough for the frontal analysis to be applied. If the binding equilibrium is rapidly established, the unbound drug concentration in the plateau region does not change even when the drug–protein separation time is prolonged. Otherwise, the drug–protein binding will not reach equilibrium state, and the concentration in the plateau zone will be lower than the true concentration of unbound drug. In other words, by increasing the separation time, the drug–protein binding will become close to the equilibrium state, resulting in the drug concentration in the plateau zone to give true unbound drug concentration. Fig. 2 compares the HPFA/CE profile of (*S*)-PRO in HDL solution under different separation time. The separation time was prolonged twofold by decreasing the applied voltage from $+4$ to $+2$ kV. No change was observed in the plateau height; the unbound PRO concentration at a lower voltage (37.8 ± 0.55 μM, $n = 3$) was the same as that determined at a higher voltage (37.6 ± 0.56 μM, $n = 3$). This result verifies that the present method is applicable to analysis of lipoprotein binding to PRO.

Further, the present HPFA/CE method was compared with a conventional ultrafiltration method. Table 4 shows the unbound concentrations of PRO enantiomers in HDL and LDL solutions. Both methods gave almost equal results. This agreement indicates the reliability of the present method. Also, HPFA/CE method has an advantage that the sample injection volume (ca. 80 nl) is much smaller than the conventional method by about two orders of magnitude.

Table 3
Total binding affinity (nK) of propranolol enantiomers to HDL and LDL

Total concentration (μM)	nK of HDL ($\times 10^4$ per M)		nK of LDL ($\times 10^4$ per M)	
	(*R*)-PRO	(*S*)-PRO	(*R*)-PRO	(*S*)-PRO
25	2.67 ± 0.069	2.62 ± 0.146	42.6 ± 1.99	42.5 ± 5.60
50	2.35 ± 0.130	2.36 ± 0.042	40.4 ± 2.51	41.1 ± 3.15
100	2.38 ± 0.037	2.27 ± 0.035	39.6 ± 2.11	39.9 ± 1.35
150	2.12 ± 0.068	2.48 ± 0.072	37.9 ± 0.66	37.4 ± 0.86
Average[a]	2.38 ± 0.217	2.43 ± 0.153	40.1 ± 2.44	40.2 ± 3.43

[a] Mean $\pm$ S.D. ($n = 3$, 12).
nK $= r$/Cu, where r and Cu represent the number of bound drug molecule per one protein molecule and unbound drug concentration, respectively.

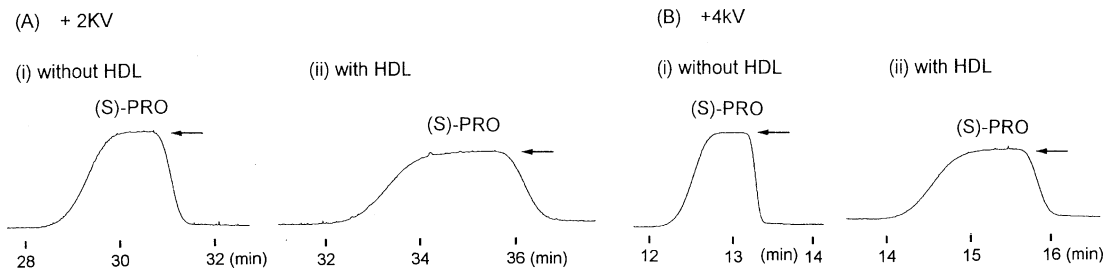

Fig. 2. Effect of applied voltage upon HPFA/CE of PRO–HDL binding. Applied voltage (A) +2 kV; (B) +4 kV. Other CE conditions, see text.

Table 4
Comparison of unbound concentrations of PRO enantiomers in lipoprotein solution measured by HPFA/CE and ultrafiltration method

Total PRO concentration (μM)	Unbound concentration of (*R*)-PRO (μM)		Unbound concentration of (*S*)-PRO	
	HPFA/CE	Ultrafiltration	HPFA/CE	Ultrafiltration
(A) In 14.6 μM HDL				
50	37.2 ± 0.53	35.4 ± 0.10	37.2 ± 0.17	34.6 ± 0.12
100	74.2 ± 0.29	73.2 ± 0.30	75.1 ± 0.29	74.6 ± 0.56
(B) in 1.90 μM LDL				
50	28.3 ± 0.77	28.0 ± 0.57	28.1 ± 0.96	27.7 ± 2.19
100	57.1 ± 1.29	61.8 ± 1.17	56.9 ± 0.82	62.8 ± 0.39

Mean ± S.D. ($n = 3$).

In conclusion, HPFA/CE method is applicable to the binding assay of plasma lipoproteins. The reliability of this method was confirmed by comparison with a conventional ultrafiltration method and by changing the drug–protein separation time. PRO enantiomers were bound to HDL and LDL in a partition-like manner, and no enantioselectivity was found. Due to the rapid analysis and small injection volume, the present system is useful for the binding study of plasma lipoproteins which are not suitable for the long-term preservation and large-scale preparation.

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