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Binding analysis of nilvadipine to plasma lipoproteins by capillary electrophoresis-frontal analysis

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Abstract

Capillary electrophoresis coupled with frontal analysis (HPCE/FA) was applied to the ultramicro analysis of enantioselective binding of nilvadipine (NV), a calcium channel blocker, to plasma lipoproteins. The drug–lipoprotein mixed solution was hydrodynamically introduced into a non-coated fused silica capillary for capillary electrophoresis. Since NV has no electric charge in the run buffer (pH 7.4), the unbound NV moved towards the cathodic end by electroosmotic flow, which was faster than the electrophoretic migrations of negatively charged lipoproteins and the bound NV. Once unbound NV migrated apart from lipoprotein, and bound NV was quickly released from the protein to maintain the binding equilibrium. Thus, NV migrated as a zone with a plateau region. The concentration of NV in this plateau region appearing on the electrophorogram was the same as the unbound NV concentration in the initial sample solution. It was found that the binding of NV to high-density lipoprotein (HDL), low-density lipoprotein (LDL) and oxidized LDL was non-specific and not enantioselective. Partition-like binding to the lipid part of these lipoproteins seemed to occur dominantly. The total binding affinities of NV to LDL were about seven times stronger than those to HDL, and the oxidation of LDL enhanced the binding affinity significantly.

Keywords: Capillary electrophoresis; Frontal analysis; Protein binding; Lipoprotein; Nilvadipine

1. Introduction

Plasma protein binding of a drug is a rapid and reversible rate process, where the concentrations of the drug and the protein are in the equilibrium state. Plasma protein binding plays an important role in pharmacokinetics and pharmacodynamics of drugs [1–3]. Since several plasma proteins contribute simultaneously to the binding of a drug, quantitative binding analysis of respective plasma protein is essential to elucidate the overall plasma distribution of the drug. Lipoproteins consist of a lipophilic core (cholesterol ester + triglycerides)

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surrounded by a surface layer comprising polar lipids (phospholipids + free cholesterol) and apolipoproteins. Plasma lipoproteins bind mainly the lipophilic neutral drugs and basic drugs [4], and act as the transport system in plasma circulation of these drugs. The binding study of plasma lipoproteins is important because considerable inter-individual differences as well as the variation depending on disease state such as coronary artery disease are found in the plasma concentrations, which possibly affect the plasma distribution of the drug.

Plasma lipoproteins are classified into several subclasses according to their density. Among them, high-density lipoprotein (HDL) and low density lipoprotein (LDL) are most important because of their high plasma concentrations. LDL suffers from in vivo oxidation. Oxidized LDL has been reported to play an important role in atherogenesis by direct cytotoxicity, by chemotactic effect on monocytes, by an inhibitory effect on macrophage motility and by initiation of foam cell formation by macrophages leading to the formation of atherosclerotic plaques that take up oxidized LDL via their scavenger receptors [5–8]. In addition, since apolipoproteins and lipid constituents such as free cholesterol, cholesterol ester and some phospholipids are chiral compounds, the binding of a racemic drug to lipoproteins may be different between the enantiomers, which can be related to enantioselective pharmacokinetic properties. Although enantioselective protein binding study is a key issue for the pharmacokinetic study of racemic drugs [9,10], the binding of a racemic drug to lipoproteins has not been investigated enantioselectively.

In previous papers, we reported high-performance capillary electrophoresis/frontal analysis (HPCE/FA) method for the analysis of plasma protein binding of basic drugs [11–14]. HPCE/FA allows binding analysis with a small sample injection volume (ca 100 nl). Since long-term preservation and large-scale preparation of lipoproteins are difficult, the HPCE/FA method is beneficial to the binding study of lipoproteins. One problem with this method arises from the adsorption of protein onto the inner surface of a fused silica capillary. Coating of the inner wall of the capillary by a hydrophilic polymer such as linear polyacrylamide is often used to avoid this problem. However, since the coating suppresses the electroosmotic flow, this approach cannot be applied to the analysis of neutral drugs. Recently we found that plasma lipoproteins are not adsorbed onto the inner surface of bare silica capillary, probably because of the negative surface charge of lipoproteins. Therefore, it is expected that HPCE/FA using a bare silica capillary can be used for the binding analysis between lipoproteins and neutral drugs.

In this study, the binding properties of nilvadipine (NV) to human HDL, LDL and oxidized LDL were investigated using the HPCE/FA method. NV, a Ca-antagonist, is clinically used as a racemate, though only the (*S*)-isomer shows pharmacological activity. NV, taking neutral form in physiological pH (7.4), is known to be bound to plasma lipoproteins [15], but the quantitative binding analysis has not been reported. In addition, NV is known to prevent oxidative modification of LDL [16], probably because the hydrophobic long chain ester at the C-5 position of dihydropyridine ring suppresses lipid radical formation from O_2 radical. However, the affinity between NV and oxidized LDL has not been studied.

2. Experimental

2.1. Materials and apparatus

A capillary electrophoresis instument 270A (Applied Biosystems) equipped with a Z-shaped non-coated fused silica capillary (122 cm × 75 μm i.d., effective length, 100 cm; LC Packings, CA), which enhances detectability, was used for HPCE/FA analysis. A Beckman L7-65 was used for ultracentrifugation and a UV-1200 UV–VIS spectrophotometer and RF-5300PC spectrofluorophotometer (Shimadzu, Japan) were used to monitor the oxidation of LDL. (*R*)- and (*S*)-NV standards were obtained from Fujisawa Pharmaceutical (Osaka, Japan). Their purities (> 99%) were confirmed by HPLC. The drug–protein mixed solutions were made up in sodium phosphate buffer solution (pH 7.4, ionic strength 0.17).

2.2. Preparation of HDL and LDL

Human HDL and LDL were prepared from plasma from a healthy male volunteer by the sequential ultracentrifugation method. Briefly, human plasma, the density of which was adjusted to 1.006 g/ml, was ultracentrifuged for 24 h × 50 000 rev./min 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 rev./min, 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 rev./min, at 4°C) gave the HDL fraction (upper fraction). In the above procedure, the density was adjusted with 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 conditions were as follows. Column, HiLoad Superdex 200pg (60 cm × 2.6 cm i.d., Pharmacia). Mobile phase, sodium phosphate buffer (pH 7.4, $I = 0.17$). Flow rate, 2 ml/min. Column temperature, 4°C. Detection, UV 254 nm. The purified HDL and LDL fractions were concentrated on the membrane (Centriplus-10, Amicon).

2.3. Oxidation of LDL

LDL was oxidized by Cu^{2+} [17]. $CuSO_4$ was added into the LDL fraction up to 5 μM. Then, the LDL fraction containing copper was shaken gently for 12 h at 37°C. After shaking, the copper ion was removed by repeated washing of the precipitate with a phosphate buffer (pH 7.4, ionic strength 0.17) at 4°C using Centriplus-10 (Amicon). The degree of oxidation was monitored by the UV absorption at 234 nm and by the fluorescence intensity (ex. 360 nm, em. 430 nm) [18].

2.4. Determination of lipoprotein concentrations

The concentrations of lipoproteins were calculated as follows. First, the concentration of apolipoprotein was measured by modified Lowry method [19] in which SDS solutions were used to prevent the interference of lipid components. Bovine serum albumin (fatty acid free) was used as the standard protein in preparing the calibration line. The molar concentration of lipoprotein was then calculated assuming that the apolipoprotein content (w/w) and the molecular weight of HDL are 50% and 1.8×10^5 Da, and those of natural and oxidized LDL are 21% and 2.3×10^6 Da, respectively [4].

2.5. Determination of unbound drug concentrations by HPCE/FA

The drug–lipoprotein mixed solution (pH 7.4, ionic strength 0.17) was introduced hydrodynamically into the capillary (4 s), and a positive voltage (+ 4 kV) was applied. The temperature was set at 25°C. The total sample volume introduced was ca 100 nl. Since NV is electrically neutral at this condition (pH 7.4), it moved towards the cathodic end (detection side) by electroosmotic flow. Unbound NV migrated faster than the negatively charged lipoprotein and the bound NV. Because drug–protein binding is reversible and kinetically rapid, their binding equilibrium can be quickly attained, while drug is separated from lipoprotein. As a result, there appears a zone of unbound NV which is detected as a trapezoidal peak having a plateau part. Thus, the unbound NV concentration can be measured from the plateau height. A series of the standard NV solutions without protein (1.80 ~ 15.8 μM of (*R*)- or (*S*)-NV in phosphate buffer, pH 7.4, 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 by 30 mM SDS and run buffer each for 1 min.

2.6. Determination of total NV concentration by HPLC

Because NV is suspected to be adsorbed onto the glass vessels, the actual total NV concentrations in the NV–lipoprotein mixed solutions in a sample vial were determined by the direct injection HPLC method. The HPLC conditions were as follows. Column, Develosil 100 diol-5 (30 cm × 8 mm i.d., Nomura Chemicals, Japan); mo-

bile phase, 40 μM NaH_2PO_4/acetonitrile (91:9); flow rate, 1.5 ml/min, detection, UV 250 nm, column temperature, 37°C. This HPLC method allowed repeatable direct sample injection analyses without deprotenization. A series of (*S*)-NV standard solutions (1–40 μM) prepared in methanol were used to obtain the calibration line. Good linearity was noted ($r = 0.9996$).

2.7. Electrophoretic mobility of lipoproteins in CZE mode

In order to confirm that protein binding equilibrium in the original sample solution remains in the sample zone in the capillary, the electrophoretic mobilities of lipoproteins in CZE mode were measured using a non-coated fused silica capillary (total length 50 cm, effective length 20 cm, inner diameter 75 μm) and a run buffer of sodium phosphate solution (pH 7.4, ionic strength 0.17) containing 0 or 20 μM (*S*)-NV. Sample solution was introduced hydrodynamically into the capillary. The applied voltage was +4 kV, and the temperature was 25°C. The lipoproteins were monitored at UV 254 nm.

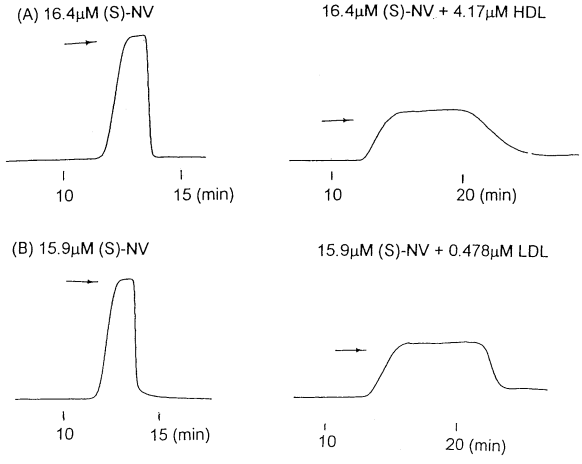

Fig. 1. HPCE/FA profiles of (*S*)-NV–lipoprotein binding. CE conditions, see text.

3. Results and discussion

Fig. 1 shows the typical electropherograms of (*S*)-NV. The left side shows the electropherograms of (*S*)-NV in protein-free solutions, where the plateau height represents the total drug concentration. The right side shows the electropherograms of (*S*)-NV in HDL solution (A) and LDL solution (B), where the concentration of (*S*)-NV was the same as in protein-free solution. The plateau heights became much lower than those of the protein-free sample solutions due to protein binding. The unbound drug concentrations were determined from these plateau heights. Similar electropherograms were obtained from (*R*)-NV in HDL and LDL solutions.

If the electrophoretic mobility of the drug–protein complex is different from that of free protein, the unbound drug concentration cannot be correctly determined, because the binding equilibrium may deviate during the electrophoretic separation, and, therefore, the drug concentration in the plateau part becomes different from the original unbound drug concentration [20]. However, this problem can be neglected when the binding does not bring about a considerable change in the protein mobility, as in the case of warfarin–albumin binding [21]. In the present study, this problem was also negligible, because the change in the electrophoretic mobility of HDL and LDL caused by the addition of NV to the run buffer in CZE mode was slight (less than 6%); the electrophoretic mobilities of HDL and LDL in the absence of NV were -0.0105 ± 0.0001 and -0.0118 ± 0.0002 cm^2/min per V, respectively, and those in the presence of 20 μM NV were -0.0111 ± 0.0002 and -0.0112 ± 0.0002 cm^2/min per V, respectively ($n = 5$).

Since lipoprotein is a molecular aggregate of apolipoproteins and several lipid components, two different binding modes would be possible. One is the binding between drug and apolipoprotein, which is site-specific as in the case of albumin and α_1-acid glycoprotein. Another is the binding of drug to lipid components, which is non-specific and partition-like. These different binding modes can be distinguished by investigating the relation between the unbound drug frac-

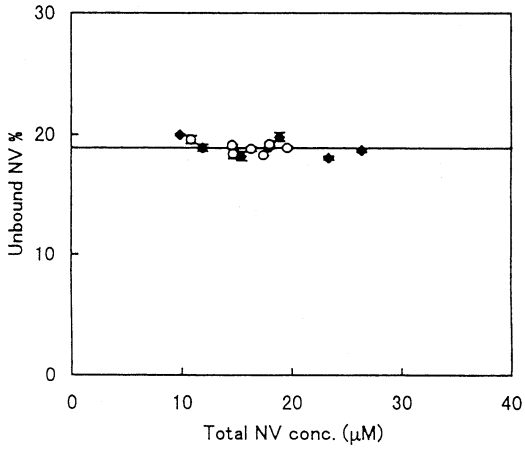

Fig. 2. Relation between total concentration and unbound fraction of (*R*)-NV (◆) and (*S*)-NV (○) in 4.17 μM human HDL solutions.

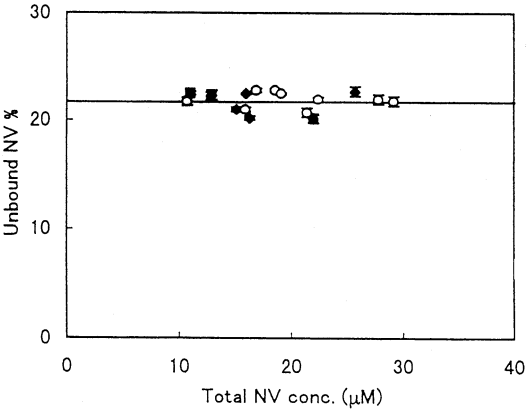

Fig. 3. Relation between total concentration and unbound fraction of (*R*)-NV (◆) and (*S*)-NV (○) in 0.130 μM native LDL solutions.

tion and total drug concentration. If the unbound drug fraction is increased with increasing total drug concentration, the specific binding mode is dominant. On the other hand, in the case where binding is non-saturable and the unbound drug fraction is constant regardless of the total drug concentration, the partition-like binding mode is dominant.

A series of sample solutions containing 9.86 ~ 26.4 μM (*R*)- or (*S*)-NV and 4.17 μM HDL were analyzed by the present method. Each sample was analyzed three times, and good reproducibility (CV < 2.11%) was obtained. Fig. 2 shows the results, where the unbound NV fractions were plotted against the total NV concentration. The unbound NV fraction was almost unchanged regardless of the total NV concentration. Apolipoproteins A-I and A-II are two major protein constituents in HDL. It is estimated that one HDL contains five to six molecules on average of these A apolipoproteins [22]. Therefore, the concentration of A apolipoproteins in the sample solutions is estimated as 21–25 μM in total. In this study, the bound NV fraction was constant even when the total NV concentration became higher than the apolipoprotein concentration. This result indicates that the NV–HDL interaction is non-specific. In addition, the unbound fractions of both enantiomers agree with each other, which indicates no enantioselectivity in NV–HDL binding. Fig. 3 shows the relation between the total concentration and the unbound fraction of NV enantiomers in LDL solutions determined by the present method. The NV concentration ranged from 10.7 to 29.2 μM, while LDL concentration remained constant (0.478 μM). Each sample was analyzed three times, and good reproducibility (CV < 2.16%) was obtained. As in NV–HDL binding shown in Fig. 2, no enantioselectivity was found in NV–LDL binding. The NV fraction is constant regardless of the total NV concentration. The main protein component found in LDL is apolipoprotein B-100. Since one LDL contains one apolipoprotein B-100 molecule [23], the maximum total NV concentration (29.2 μM) was about 60 times higher than the apolipoprotein concentration (0.478 μM). This result strongly suggests that the partition-like binding to the lipid part seems to be dominant in NV–HDL binding and NV–LDL binding rather than specific binding to apolipoprotein.

Frontal analysis is applicable to the analysis of binding equilibrium which is rapidly established, like binding of drug to albumin or α_1-acid glycoprotein. If the drug–protein binding proceeds much more slowly than the separation, the drug in the plateau region will not reflect the actual concentration of the unbound drug. Unlike albumin or α_1-acid glycoprotein, to which drug is bound stoichiometrically at specific binding

site(s), lipoproteins shows non-specific and partition-like binding character. Therefore, it is important to confirm if the drug binding to lipoprotein occurs rapidly enough for the frontal analysis. For this purpose, the same sample was analyzed under the different applied voltages (+ 2 and + 4 kV). The drug–protein separation time under the applied voltage of + 2 kV is about twofold longer than that under the applied voltage of + 4 kV. If the binding equilibrium is rapidly established, the drug concentration in the plateau region should be independent of the applied voltage. Otherwise, it is expected that the drug concentration in the plateau region will be different when measured under the different applied voltages. In other words, the binding condition will approach closely to the equilibrium state by applying the lower voltage because of the longer separation time. When we determine the unbound concentration in 17.4 μM (*S*)-NV and 0.478 μM LDL mixed solution, the same result was obtained under the different applied voltages; the unbound NV concentration measured at the lower voltage was 3.66 ± 0.07 μM ($n = 3$), while that measured at the higher voltage was 3.72 ± 0.001 μM ($n = 3$). This result indicates that NV binding to lipoprotein occurred rapidly enough for the experimental condition of the present frontal analysis to be applicable to the lipoprotein binding analysis.

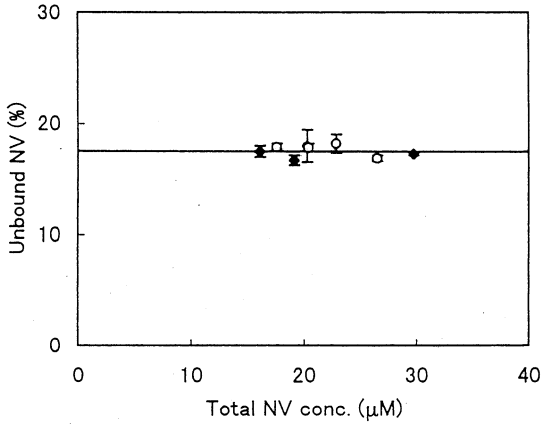

Fig. 4. Relation between total concentration and unbound fraction of (*R*)-NV (◆) and (*S*)-NV (○) in 0.130 μM oxidized LDL solutions.

Table 1
Total binding affinity (nK) between NV enantiomers and lipoproteins[a]

	nK (M^{-1})	
	(*R*)-NV	(*S*)-NV
HDL	$1.02\ (\pm 0.048) \times 10^6$	$1.02\ (\pm 0.027) \times 10^6$
Normal LDL	$7.66\ (\pm 0.529) \times 10^6$	$7.47\ (\pm 0.324) \times 10^6$
Oxidized LDL	$3.66\ (\pm 0.131) \times 10^7$	$3.57\ (\pm 0.135) \times 10^7$

[a] Values are mean ± S.D. Number of analyses (n = 21 (HDL), 27 (normal LDL), 16 (oxidized LDL)).

LDL oxidation is accompanied with the change in UV absorption at 234 nm due to the formation of conjugated diene in the lipid phase [24] and with the increase in fluorescence intensity (ex. 360 nm, em. 430 nm) due to Schiff base formation by the reaction of the ε-amino group of Lys residue in apoprotein B with aldehydes or hydroperoxides which are the degradation products from lipid peroxidates of unsaturated fatty acids composed of esterified cholesterol and phosphatidylcholine [25]. After the 12-h oxidation by copper ion, the UV absorbance of 0.019 μM LDL solution was increased from 0.095 to 0.187, and the fluorescence intensity changed from 1.57 to 13.2 (arbitrary units). This means that both lipids and apolipoprotein suffered from the oxidation. The electrophoretic mobility of LDL changed from -0.0118 to -0.0136 cm^2/min per V, which indicates the increase in negative net charge.

The mixed solutions containing 16.1 ~ 29.8 μM NV and 0.130 μM oxidized LDL were analyzed by HPCE/FA method, and the unbound NV fractions were calculated. The results are shown in Fig. 4. As in the case of native LDL, against the increase in the total NV concentration, the unbound NV fraction did not change, indicating non-saturable and partition-like binding character. In addition, no enantioselectivity was observed.

Table 1 shows the total binding affinity (nK) of NV enantiomers to HDL, LDL and the oxidized LDL calculated from the data in Figs. 2–4, respectively. The total binding affinity of LDL containing higher lipid fraction (ca 80%) than HDL

(ca 50%) was 7.6 times higher than that of HDL. This result supports that the binding to the lipid phase is dominant in NV–lipoprotein binding. The oxidation increased the drug affinity of LDL markedly; the nK value of NV–oxidized LDL binding was about 20 times stronger than that of native LDL.

In conclusion, it was demonstrated that the HPCE/FA method is applicable to the study of strong binding between lipoproteins and a neutral drug. NV is bound to HDL, LDL and oxidized LDL in non-specific and partition-like manner, and no enantioselectivity was observed. LDL with higher lipid content shows stronger affinity than HDL, and the oxidation of LDL increases the binding affinity by about 20 times. Since the present method requires only a small sample injection volume, it is beneficial to the binding study of lipoproteins and oxidized lipoprotein, because their large-scale preparation and long-term preservation are difficult.

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