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Stereoselective Plasma Protein Binding of Amlodipine

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ABSTRACT The binding of the (R)- and (S)-enantiomers of amlodipine to bovine serum albumin (BSA), human serum albumin (HSA), α_1-acid glycoprotein (AGP), and human plasma (HP) was studied by equilibrium dialysis over the concentration range of 75–200 μM at a protein concentration of 150 μM. Unbound drug concentrations were determined by enantioselective capillary electrophoresis using 50 mM phosphate buffer, pH 2.5, containing 18 mM α-cyclodextrin as background electrolyte. Saturation of the protein binding sites was not observed over the concentration range tested. Upon application of racemic amlodipine besylate, (S)-amlodipine was bound to a higher extend by HSA and HP compared with (R)-amlodipine, whereas the opposite binding of the enantiomers was observed for BSA and AGP. Scatchard analysis was used to illustrate the different binding affinities of amlodipine besylate enantiomers to BSA, HSA and AGP. *Chirality 22:262–266, 2010.* © 2009 Wiley-Liss, Inc.

KEY WORDS: Amlodipine; enantiomer; protein binding; capillary electrophoresis

INTRODUCTION

It is well established that the enantiomers of drugs may exhibit different pharmacologic and pharmacokinetic properties.[1] This includes the stereoselective binding of drugs to plasma protein due to the inherent chiral nature of proteins.[2] As such stereoselective interactions may affect the pharmacokinetics of drug enantiomers, protein binding studies are an integral part in the development of chiral drugs.

Amlodipine (3-ethyl-5-methyl-2-(2-aminoethoxymethyl)-R,S-4-(2-chlorophenyl)-1,4-dihydro-6-methyl-3,5-pyridinedicarboxylate) is a chiral dihydropyridine calcium antagonist used as the racemate for the treatment of hypertension and ischemic heart disease with distinctive pharmacokinetic characteristics which appear to be attributed to a high degree of ionization.[3–5] As reported for other dihydropyridines[6] the pharmacological activity is associated with the (S)-(−)-enantiomer.[7] The drug displays an oral bioavailability of 50–80% with peak plasma concentrations at about 6–8 h. Amlodipine is extensively metabolized in the liver and slowly cleared with a terminal half live of 40–50 h.[4,8,9] After administration of the enantiomers of amlodipine as well as racemic amlodipine to healthy male volunteers, comparable pharmacokinetic parameters were reported for the amlodipine enantiomers but somewhat higher plasma concentrations were observed for the (S)-enantiomer.[10] In contrast, higher plasma concentrations of (R)-amlodipine than its (S)-enantiomer were reported in elderly hypertensive patients.[11]

With regard to the stereoselective protein binding of the amlodipine enantiomers, inconclusive results have been published. Kubicek and Laznickova[12] studied the binding of the individual enantiomers to human plasma (HP), human serum albumin (HSA) and α_1-acid glycoprotein (AGP) by equilibrium dialysis followed by non-stereoselective HPLC analysis. Stronger binding of (S)-amlodipine to human plasma was observed, whereas the (R)-enantiomer was bound stronger by AGP and there was no difference between the enantiomers in the case of HSA. Employing a frontal analysis capillary electrophoresis assay, Zhao et al.[13] did not observe a difference of the binding of racemic amlodipine and (S)-amlodipine to HSA or bovine serum albumin (BSA). None of these reports employed a stereoselective analytical method. Therefore, the present study was performed in order to analyze the protein binding of the amlodipine enantiomers upon equilibrium dialysis using racemic drug and (S)-amlodipine.

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EXPERIMENTAL

Chemicals

Racemic amlodipine besylate and (S)-amlodipine besylate were provided by Smruthi Organics Ltd (Solapur, India). Fatty acid free HSA, BSA, and fatty acid and globulin free AGP were purchased from Sigma-Aldrich (Schnelldorf, Germany). HP was supplied by the Institute of Transfusion Medicine, Friedrich-Schiller-University Jena, Germany. α-Cyclodextrin was from Cyclolab (Budapest, Hungary). All other chemicals were of analytical grade

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and used without further purification. Buffer solutions were prepared with deionized, double distilled water.

Capillary Electrophoresis

All experiments were performed using a Biofocus 3000 capillary electrophoresis instrument (Biorad, Munich, Germany) equipped with a 50/46 cm, 50 μm i.d. fused-silica capillary operated at 18°C. The optimized background electrolyte consisted of 50 mM sodium phosphate buffer, pH 2.5, containing 18 mM α-cyclodextrin. The applied voltage was 20 kV. UV detection was performed at 214 nm. Samples were introduced at a pressure of 20 psi for 4 s. Before the analyses the capillary was successively rinsed with 0.1 M NaOH for 15 min, water for 15 min, 0.1 M HCl for 15 min and the background electrolyte for 15 min. Between analyses, the capillary was washed with 0.1 M NaOH for 2 min and the background electrolyte for 5 min.

Equilibrium Dialysis

A Dianorm dialysis unit (Bachofer Laboratories, Reutlingen, Germany) consisting of teflon microcells with two 1 ml chambers separated by a semipermeable membrane (12,000 molcular weight cut off) was thermostated at 36°C. Stock solutions of racemic amlodipine besylate, (S)-amlodipine besylate and solutions of BSA, HSA and AGP were prepared in 50 mM sodium phosphate buffer, pH 7.4 and stored in the dark in a refrigerator at 4–8°C. Appropriate amounts of the respective solutions were mixed to yield a final concentration of 150 μM of the proteins and 75, 100, 150, and 200 μM of racemic amlodipine besylate and (S)-amlodipine besylate, respectively. HP was used as obtained. The protein containing drug solutions were placed in the donor compartment, whereas protein and compound free buffer was placed in the receiving compartment. Following an equilibration time of 14 h the drug in the receiving compartment was analyzed by enantioselective capillary electrophoresis. Each experiment was performed six times. The bound drug concentrations were calculated from the concentration of the free drug, F, and the total drug concentration, T, according to

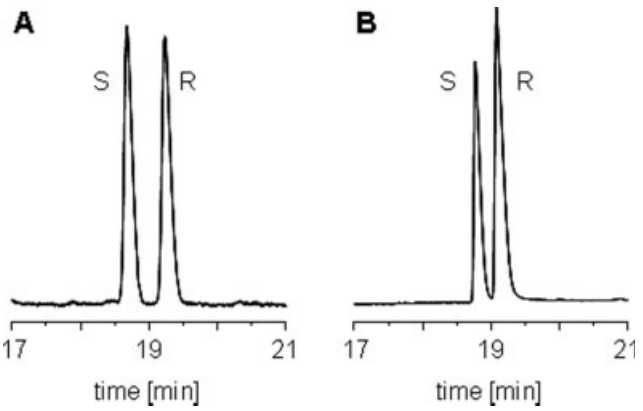

Fig. 1. Electropherograms of (**A**) racemic amlodipine and (**B**) incubation of 150 μM racemic amlodipine in the presence of 150 μM HSA. For experimental conditions, see experimental part.

$$\text{bound drug } [\%] = \frac{T - F}{T} \cdot 100 \quad (1)$$

The binding parameters were determined according to[14]

$$\frac{r}{c_u} = -Kr + nK \quad (2)$$

where r is the number of mols of bound drug per mol protein, c_u is the free drug concentration, K is the binding constant and n the number of binding sites.

RESULTS

Enantioselective Capillary Electrophoresis

The separation of the enantiomers of amlodipine by capillary electrophoresis has been achieved using uncharged[15,16] and negatively charged cyclodextrin derivatives.[15] Following an initial screening using various neutral and sulfated cyclodextrin derivatives, α-cyclodextrin was selected and a concentration of 18 mM in a 50 mM phosphate buffer, pH 2.5, gave a baseline separation with a resolution of $R_S = 2.3$. (S)-Amlodipine (18.7 min) migrated before the (R)-enantiomer (19.4 min) (Fig. 1A).

TABLE 1. Protein binding of amlodipine enantiomers in racemate and of (S)-amlodipine to BSA, HSA, HP, and AGP. The data are expressed as percent bound drug (mean ± SD, $n = 6$)

Concentration (μM)	Enantiomer	BSA	HSA	HP	AGP
75	(R)-AML, racemate	97.6 ± 0.1	91.8 ± 0.6	93.8 ± 0.1	58.4 ± 0.2
	(S)-AML, racemate	94.9 ± 0.2	97.5 ± 0.3	96.2 ± 0.5	55.7 ± 0.3
	(S)-AML	97.0 ± 0.3	95.1 ± 0.3	95.0 ± 0.3	53.5 ± 0.3
100	(R)-AML, racemate	95.6 ± 0.1	87.8 ± 0.3	90.6 ± 0.2	57.3 ± 0.6
	(S)-AML, racemate	92.2 ± 0.4	93.4 ± 0.7	94.4 ± 0.4	53.8 ± 0.6
	(S)-AML	95.9 ± 0.3	94.4 ± 0.5	94.3 ± 0.3	52.1 ± 0.7
150	(R)-AML, racemate	93.6 ± 0.1	85.6 ± 0.4	87.5 ± 0.4	56.5 ± 0.7
	(S)-AML, racemate	88.7 ± 0.4	90.6 ± 0.4	91.1 ± 0.2	52.7 ± 0.2
	(S)-AML	93.1 ± 0.5	92.9 ± 0.2	91.7 ± 0.3	50.8 ± 0.4
200	(R)-AML, racemate	91.8 ± 0.1	84.0 ± 0.4	85.6 ± 0.2	53.9 ± 0.8
	(S)-AML, racemate	87.7 ± 0.7	89.6 ± 0.7	89.8 ± 0.3	50.8 ± 0.6
	(S)-AML	89.8 ± 0.5	90.8 ± 0.2	91.0 ± 0.3	49.0 ± 0.6

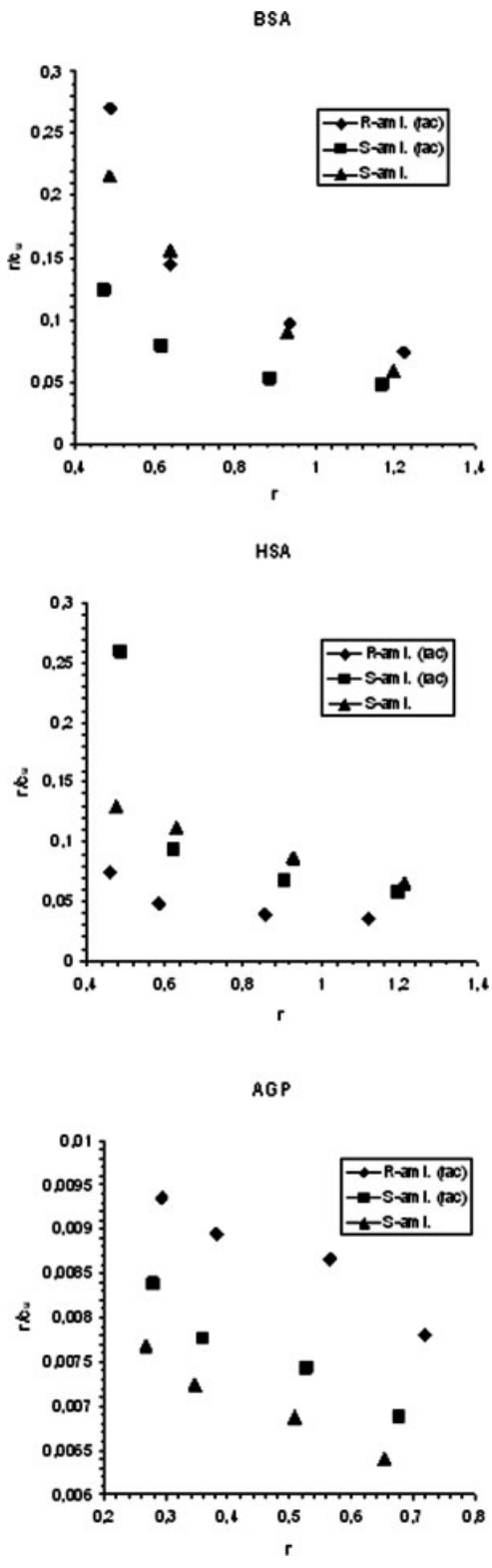

Fig. 2. Scatchard plots of the protein binding of (R)-amlodipine (◆) and (S)-amlodipine (■) from racemic drug and of (S)-amlodipine (▲). The data points are expressed as mean of six separate experiments, the standard deviations are smaller than the symbols.

The assay was subsequently validated using racemic amlodipine with respect to linearity, range, limit of quantitation, limit of detection and precision. The assay was linear for both enantiomers over the range 2–120 μM (4–240 μM racemic amlodipine) with regression coefficients of at least $r^2 = 0.998$. The limit of quantitation (signal to noise ratio of 10) was 2 μM for both enantiomers, whereas the limit of detection (signal to noise ratio of 3) was 0.8 μM for (S)-amlodipine and 1 μM for (R)-amlodipine. Precision of the assay was determined at concentrations of 2 and 100 μM by injecting the samples six times on one day and three times on three consecutive days. The relative standard deviation varied between 0.4 and 4.1% (data not shown) indicating good reproducibility of the data. Moreover, no degradation of amlodipine was observed upon storage for 14 h in the dark at 36°C indicating the stability of the compound under the experimental conditions of the equilibrium dialysis experiment.

Drug Protein Binding

The results for the binding of the amlodipine enantiomers by HSA, BSA, AGP and HP as determined by equilibrium dialysis of the proteins in the presence of racemic amlodipine besylate or (S)-amlodipine besylate are summarized in Table 1 and Figure 2. Figure 1B shows an electropherogram of the unbound amlodipine enantiomers in an incubation of 150 μM racemic amlodipine besylate in the presence of 150 μM HSA. For all proteins, binding decreased with increased concentration of amlodipine (Table 1). Although about 97.6–93.8% [depending on the enantiomer and the applied form (racemate or (S)-enantiomer)] were bound with incubation conditions of 75 μM of the drugs and 150 μM protein by BSA, HSA or HP, the values decreased to 91.8–84.0% at a drug concentration of 200 μM. AGP displayed a much weaker binding for the drug with only about 50–60% bound under these experimental conditions. The binding of amlodipine by the proteins was not saturated in the concentration range tested as can be seen from Figure 2. HSA displayed stereoselectivity for the enantiomers, with (S)-amlodipine being bound stronger compared with the (R)-enantiomer when applying the racemate. In contrast, BSA and AGP showed opposite stereoselectivity, the concentration of free (S)-enantiomer always exceeding the concentration of (R)-amlodipine. HP displayed comparable stereoselectivity to HSA.

Specific binding constants for the individual proteins HSA, BSA and AGP could not be derived from the Scatchard plots (see Fig. 2) because of the biphasic nature of the curves. They were estimated to be in the range of reported values[13] in case of the specific binding region.

DISCUSSION

The binding of the enantiomers of amlodipine upon incubation of racemate and pure (S)-amlodipine to plasma proteins was studied by equilibrium dialysis. A stereoselective CE method for amlodipine enantiomers was developed and validated based on published procedures. Thus, Owens et al.[15] separated the amlodipine enantio-

mers using neutral cyclodextrin derivatives such as hydroxypropyl-β-cyclodextrin or the charged derivatives carboxymethyl-β-cyclodextrin and sulfobutylether-β-cyclodextrin, whereas Small et al.[16] employed α-cyclodextrin. In our hands, α-cyclodextrin proved to be a preferable chiral selector compared with other cyclodextrins tested. Compared with Small et al.[16] a lower pH of the background electrolyte was selected leading to shorter analysis times.

Previous studies have analyzed either the binding of the individual enantiomers using a non-stereoselective HPLC assay[12] or compared the total binding of racemic drug and the (S)-enantiomer by non-stereoselective CE frontal analysis.[13] Neither of these assays allowed to study the influence of the (R)-enantiomer on the protein binding of the (S)-enantiomer.

Racemate and (S)-amlodipine are strongly bound to purified serum albumin, i.e., BSA and HSA, and also to HP but to a lesser extend to AGP. This is consistent with reported data.[12] Amlodipine also displayed high protein binding in pharmacokinetic studies.[9]

The amlodipine enantiomers were bound to plasma proteins in a stereoselective manner. Generally, HSA[2] and AGP[17] are well known to bind drugs stereoselectively and this has also been reported for other dihydropyridine calcium antagonists.[18,19] Interestingly, opposite chiral selectivity was observed for BSA and AGP on one hand and HSA on the other hand. Kubicek et al. also reported the opposite stereoselectivity of the binding of the amlodipine enantiomers by HP and AGP with the (S)-amlodipine bound more strongly to HP, whereas (R)-amlodipine interacted stronger with AGP.[12] However, in contrast to the present results, no difference was observed for the binding of the individual enantiomers to isolated HSA by Kubicek and Lanznickova.[12] Opposite stereoselectivity for the enantiomers of HSA and AGP towards the dihydropyridine derivative isradipine has been reported, with the (R)-enantiomer binding more strongly to AGP but weaker to HSA compared with the (S)-enantiomer.[18] HSA and HP showed essentially comparable selectivity but a smaller effect was noted for HP. This may be explained by the fact that HP contains not only HSA but also other proteins such as globins or AGP. A different stereoselectivity of the binding of the amlodipine enantiomers has been shown for AGP in the present study.

When comparing the binding of (S)-amlodipine alone and in the presence of (R)-amlodipine (incubation with the racemate) an effect of the (R)-enantiomer on the binding of the (S)-isomer was noted specifically for BSA (Fig. 2 and Table 1). This may be due to a competition for the binding sites on the protein. Concentration-dependent changes for amlodipine binding to HP were not significant. This may be due to the presence of other proteins such as AGP in HP with different binding mechanisms (acid-base interactions) compared with HSA and, moreover, different selectivities for the complexation of the amlodipine enantiomers. The binding to globulins was not investigated but may also be different. The stronger binding of (S)-amlodipine upon incubation of 75 μM racemic drug with HSA is contrary to the generally observed trend. There is no apparent explanation for this phenomenon except that only very low concentrations of the free drug were measured so that the data may not be significantly different. At 200 μM, a reduction of stereoselectivity was observed for BSA and HSA. It may be speculated that the unspecific binding of the enantiomers to the albumins at high concentrations is non-selective and, therefore, diminishes the effect observed at lower concentrations.

The smaller amounts of free amlodipine in the case of human plasma may have been caused by the participation of other proteins like globulins or AGP which are present in plasma in addition to albumin. As shown in Figure 2, the descending parts of the BSA- and HSA-curves indicated the presence of approximately one specific saturable binding site. The flattening of the curves at higher drug concentrations demonstrate the non-saturable binding of amlodipine caused for example by lipophilic or ionic interactions (log D_{base} = 2.20 at pH 7.0[20]) with certain protein domains. This finding was verified in the same manner by Scatchard plots for each enantiomer using HSA and BSA as model proteins. AGP binding tended to be slightly stereospecific concerning (S)-amlodipine, but the shape of the AGP/amlodipine plots did not allow the calculation of specific binding constants.

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