Enantioselective Retention of 4-Aryl-1,4-dihydropyridine Calcium-Channel Blockers on Human Serum Albumin and α_1-Acid Glycoprotein HPLC Columns: Relationships with Different Scales of Lipophilicity

by **Francesco Barbato***, **Fabiana Quaglia**, **Maria Tiziana Quercia**, and **Maria Immacolata La Rotonda**

Dipartimento di Chimica Farmaceutica e Tossicologica, Facoltà di Farmacia, Università degli Studi di Napoli Federico II, Via D. Montesano, 49, I-80131 Naples
(tel. +39 081 7486627; fax +39 081 7486630; e-mail: fbarbato@unina.it)

The enantioselective retention of eight 4-aryl-1,4-dihydropyridine (DHP) calcium-channel blockers on HPLC stationary phases supporting human serum albumin (HSA) or α_1-acid glycoprotein (AGP) was investigated. All chiral neutral DHPs were resolved on the AGP column, whereas, on the HSA column, only isradipine showed a split chromatographic peak. Analyses performed on AGP with eluents containing dimethyloctylamine (DMOA) as the displacer demonstrated that the protein has at least two binding sites for DHPs. The first family of binding sites is enantioselective, binds exclusively to the (*R*)-forms, and presumably interacts competitively with DMOA. The second family of binding sites appears to be non-enantioselective and is affected by a cooperative interaction with DMOA. For the selected set of DHPs, the lipophilicity scale in octan-1-ol/H_2O (log *P*) was not collinear with log k_w^{IAM} values obtained with immobilized artificial membranes (IAM-HPLC) due to the inclusion of both neutral and basic congeners. Only for the neutral DHPs did log k_w^{IAM} behave as a better descriptor than log *P* for retention data on HSA and AGP. In fact, the behavior of the basic DHPs amlodipine and nicardipine on both proteins correlated better with the octan-1-ol/H_2O log *P* values. We, therefore, infer that the amphipathic nature of the IAM phase only mimics the interaction of non-ionizable compounds with serum proteins. In contrast, the IAM-HPLC retention data of protonated bases encode additional interaction mechanisms that are specific for phospholipids and not involved in ligand-protein interactions.

Introduction. – Serum-protein binding strongly affects the pharmacokinetic behavior of drugs, the two major drug-binding proteins being human serum albumin (HSA) and α_1-acid glycoprotein (AGP). Although earlier studies reported that drugs can bind to serum proteins by essentially nonspecific partition-based mechanisms, it was later demonstrated that drug-binding processes involve specific domains of proteins able to recognize enantiomers [1][2]. This is one of the mechanisms that account for the different pharmacokinetic behavior of enantiomers and make them useful probes for investigating drug-protein interactions. However, the usual biochemical methods to assess protein binding are time-consuming and require substantial amounts of pure, isolated enantiomers. Besides, racemization processes can become a complicating factor.

The study of drug-protein interactions can be facilitated by HPLC methods based on stationary phases packed with serum proteins chemically immobilized on a silica-gel core (biochromatography) [3]. This technique yields binding-related retention data that mirror the drug-binding properties of the corresponding free protein. This is clearly the case for HSA [3–9], whereas for AGP there was some dispute as to whether chromatographic data correlate with biochemical binding data [10][11]. However,

recent evidence confirms that AGP columns can yield retention data that correlate with protein binding data [11][12]. The HPLC methods have the advantage of producing quantitatively comparable binding-related retention data for large sets of compounds. Furthermore, they allow the affinity of enantiomers to be assessed even without prior resolution or in the event of racemization.

HSA is the most important drug-binding serum protein, with a pronounced affinity for acidic and neutral compounds. At least two major sites have been reported to enantioselectively bind drugs, namely 'site I' which binds warfarin and azapropazone, and 'site II' which binds profens and benzodiazepines [13][14]. There is evidence of further enantioselective binding sites for other classes of drugs [4][15]. AGP has a pronounced affinity for basic drugs [16], but few studies have examined its affinity towards acidic and uncharged compounds [17][18]. However, it seems that the structural requirements of neutral compounds for separation on AGP columns are analogous to those of charged solutes, namely that the compounds should have two H-bonding groups and a rigid or bulky structure close to the center of chirality [17][19]. This is the case for the dihydropyridine calcium-channel blockers (DHPs). These compounds are neutral (the 4-aryl-1,4-dihydropyridine moiety is neither acidic nor basic in aqueous media), unless an amino function is present as in amlodipine[1]) and nicardipine [1]). This renders their resolution extremely difficult and expensive because the available chemical processes (*e.g.* crystallization) are not suitable. As a result, DHPs are marketed as racemates although it is known that the two enantiomers have different pharmacological potencies, the (*S*)-forms being mainly responsible for activity [20]. Moreover, the possible co-administration of other drugs that bind to the same enantioselective site of a serum protein could generate a pharmacokinetic interaction involving displacement of DHP from the protein and an increase in the serum levels of its unbound form.

The present study examines eight DHPs, namely six neutral (nifedipine[1]), nitrendipine[1]), nimodipine[1]), isradipine[1]), felodipine[1]), and nisoldipine[1])) and two basic drugs (amlodipine[1]) and nicardipine[1])). All compounds except nifedipine are chiral. We investigated the experimental conditions that allow the chromatographic resolution of the racemic mixtures on both HSA and AGP columns. A separation of enantiomers would establish that specific enantioselective binding sites exist in the proteins. We also investigated the existence of possible relationships between chromatographic capacity factors of the proteins and various lipophilicity parameters, namely log *P* (octan-1-ol/H_2O partition coefficients determined by the 'shake-flask' procedure) [21] and log k_w^{IAM} (chromatographic capacity factors measured by HPLC on a phospholipid-based stationary phase, the so-called immobilized artificial membrane

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[1]) Amlodipine = ethyl methyl 2-[(2-aminoethoxy)methyl]-4-(2-chlorophenyl)-1,4-dihydro-6-methylpyridine-3,5-dicarboxylate; nicardipine = methyl 2-[methyl(phenylmethyl)amino]ethyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate; nifedipine = dimethyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylate; nitrendipine = ethyl methyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-pyridine-3,5-dicarboxylate; nimodipine = 2-methoxyethyl 1-methylethyl 1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate; isradipine = methyl 1-methylethyl 4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-2,6-dimethylpyridine-3,5-dicarboxylate; felodipine = ethyl methyl 4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethylpyridine-3,5-dicarboxylate; nisoldipine = methyl 2-methylpropyl 1,4-dihydro-2,6-dimethyl-4-(2-nitrophenyl)pyridine-3,5-dicarboxylate.

(IAM), extrapolated to 100% aqueous phase) [22–24]. The latter parameters were considered on the basis of our previous work on nine DHPs [25], including the present compounds. In fact, we found that the log *P* lipophilicity scale was not collinear with $\log k_w^{IAM}$, mainly due to the particular behavior of the two basic DHPs. We also observed that biological phenomena that include interactions with biomembranes correlated better with $\log k_w^{IAM}$ than with log *P*. Moreover, *Kaliszan* and co-workers [11][12] found that the interactions of 16 antihistamines and 7 β-blockers on an AGP phase were better related to $\log k_w^{IAM}$ than to log *P*. These observations suggest that the amphipatic nature of phospholipids allows them to be a more effective partition phase than octan-1-ol for describing partitioning in biomembranes as well as in other biological components such as proteins.

In the present study, we also used the displacement-chromatography technique to characterize enantioselective binding sites. Displacement chromatography is based on interactions between solutes that bind at a common binding site. A competitive solute (displacer) is added to the mobile phase, and the effect on the retention of ligands is observed. Studying the variations in ligand elution at various percentages of the displacer can provide very useful information on the identity of binding sites for different classes of drugs.

Experimental. – *Materials.* All samples were obtained from commercial sources. The chemicals were of HPLC grade and used without further purification. Samples of the pure enantiomers (+)-(*R*)-nimodipine, (−)-(*S*)-nimodipine, (−)-(*R*)-nisoldipine, (+)-(*S*)-nisoldipine, and (+)-(*R*)-nitrendipine were kindly provided by *Bayer Italia S.p.A.*, Milano, Italy.

Chromatographic System and Conditions. A *600-E* liquid chromatograph (*Waters-Millipore*, Milford, MA) equipped with a *7125-Rheodyne* injection valve (fitted with a 20-µl loop) and a *486* UV detector (*Waters*) set at λ 238 nm was used. The stainless steel columns were HSA (4.6 × 150 mm; *Shandon*, Runcom, UK) and *Chiral AGP 100* (4.0 × 100 mm; *Chromtech AB*, Hägersten, Sweden). The chromatograms were recorded by a *Millipore-746* data module. HSA chromatography: MeCN/0.05M phosphate-buffered saline (PBS) at pH 7.0 15 : 85 (*v*/*v*); flow rate 0.5 ml/min. AGP chromatography: mixtures of propan-2-ol/0.01M phosphate buffer saline (PBS) at pH 7.0, in percentages ranging from 10 to 20% (*v*/*v*); flow rate 0.9 ml/min. In the analyses with the displacer, the concentrations of *N*,*N*-dimethyloctylamine (DMOA) were 1.0, 2.0, 3.0, and 4.0 mM.

Eluent mixtures were obtained directly by mixing at low pressure the org. modifier and the aq. phase, which were previously degassed by bubbling with He. Chromatography was carried out at r.t. Samples were dissolved in the mobile phase (*ca.* 10^{-4} M), and 20 µl of the sample were injected. Chromatographic retention data are expressed by the logarithm of the capacity factor, log *k*′, defined as $\log k' = \log [(t_R - t_0)/t_0]$, where t_R and t_0 are the retention times of the drug and a non-retained compound (MeCN), resp. All values of log *k*′ were the average of at least three measurements; the 95% confidence interval associated with each value never exceeded 0.04.

Lipophilic Parameters. Both octan-1-ol/buffer partition coefficients (log *P*) and chromatographic capacity factors on the IAM stationary phase ($\log k_w^{IAM}$) were taken from our previous work [25].

Determination of the Absolute Configuration Following HPLC Separation on the AGP Column. The absolute configurations of the enantiomers generating the respective chromatographic peaks were determined as follows: For nitrendipine, nisoldipine, and nimodipine, the chromatographic peaks of the racemic mixture were compared with those obtained under the same conditions for the pure enantiomers and for known mixtures thereof; for both isradipine and felodipine, the fractions containing the pure enantiomers were collected at the outlet of the chromatograph, and the optical rotation was measured at 589.3 nm (*Polax-D* polarimeter, *Atago*, Japan). (*R*)-Isradipine and (*S*)-felodipine are known to be levorotatory [26][27].

Results and Discussion. – *HSA Chromatography.* The analyses were performed at pH 7.0 to achieve near-physiological conditions compatible with the stability of the stationary phase. Eluents with different ionic strengths and MeCN percentages did not result in the separation of enantiomers. The best separations, *i.e.* allowing elution of all compounds within *ca.* 30 min, were achieved with MeCN/0.05M phosphate buffer pH 7.0 15:85 (*v*/*v*). Under these conditions, only isradipine showed two peaks. These results are compatible with the existence on HSA of enantioselective binding sites for DHPs, but only if these sites play a marginal role in the overall retention of DHPs, implying that the latter is mainly determined by nonspecific lipophilic interactions.

To gain further insight into the latter mechanism, we compared the chromatographic data on HSA with octan-1-ol/H_2O partition coefficients ($\log P$) and with affinity data by the IAM-HPLC method ($\log k_w^{IAM}$) [25]. The $\log D_{7.4}$ values were also considered for the two basic DHPs, *i.e.* their octan-1-ol/H_2O distribution coefficient at pH 7.4, where both ionized and neutral forms contribute to the partitioning.

The $\log P$ and $\log k_w^{IAM}$ values are not collinear for the whole set of eight DHPs, but they are interrelated when only the six neutral DHPs are considered (*Eqn. 1*). *Eqn. 1* indicates that for neutral DHPs, *ca.* 90% of the variation in $\log k_w^{IAM}$ depends on the variation in $\log P$. As a consequence, a similar correlation was expected between $\log k'$ on HSA (*Table 1*) and either $\log k_w^{IAM}$ or $\log P$. This deduction is, indeed, verified (see *Eqns. 2* and *3*).

$$\log k_w^{IAM} = 0.753\ (\pm 0.126) \cdot \log P - 0.796\ (\pm 0.531) \quad n = 6\ r = 0.948\ s = 0.151 \tag{1}$$

$$\log k'_{HSA} = 0.557\ (\pm 0.060) \cdot \log k_w^{IAM} - 0.418\ (\pm 0.143) \quad n = 6\ r = 0.977\ s = 0.057 \tag{2}$$

$$\log k'_{HSA} = 0.427\ (\pm 0.076) \cdot \log P - 0.891\ (\pm 0.391) \quad n = 6\ r = 0.942\ s = 0.090 \tag{3}$$

Table 1. *Lipophilic Parameters and Retention Data of DHPs on HSA*

	$\log P$ ($\log D_{7.4}$)[a])	$\log k_w^{IAM}$ [a])	$\log k'_{HSA}$ [b])
Nifedipine	3.22	1.74	0.56 (± 0.01)
Nitrendipine	4.15	2.27	0.84 (± 0.02)
Nimodipine	4.18	2.35	0.80 (± 0.02)
Isradipine	4.18	2.13	0.83 (± 0.02)
Nisoldipine	4.53	2.63	1.05 (± 0.02)
Felodipine	4.80	2.98	1.27 (± 0.03)
Amlodipine	3.30 (1.83)	2.59	0.57 (± 0.02)
Nicardipine	4.96 (3.72)	3.14	0.60 (± 0.02)

[a]) Data taken from [25]. [b]) Logarithm of capacity factor on HSA column (± s.d.); eluent: MeCN/0.5M phosphate buffer pH 7.0 15:85 (*v*/*v*); flow rate 0.5 ml/min; $n \geq 3$.

The behavior of the basic compounds amlodipine and nicardipine is difficult to rationalize. The inclusion of the $\log k_w^{IAM}$ value of amlodipine in *Eqn. 2* yielded a much less significant relation ($r = 0.723, s = 0.104$), whereas the inclusion of nicardipine led to

a total loss of significance ($r = 0.340$; $s = 0.172$). This indicates that the log k_w^{IAM} values of basic compounds are poor descriptors of their binding to HSA, in contrast to their interactions with biomembranes [25]. Indeed, log k_w^{IAM} values are composite parameters that do not adequately mirror the polar and ionic interactions between HSA and bases, but that mirror the various binding interactions with biomembranes. In contrast to log k_w^{IAM}, the log P parameter adequately describes interactions of basic DHPs with HSA, with the proviso that log $D_{7.4}$ may be an even better parameter. Thus, by taking the log P value (but not the log $D_{7.4}$) of amlodipine, and the log $D_{7.4}$ (but not the log P) of nicardipine, one obtains a slightly improved correlation ($n = 8$, $r = 0.945$, $s = 0.045$).

A possible explanation could be related to the different binding properties of compounds having a primary *vs.* tertiary amino function (amlodipine *vs.* nicardipine). It is well known that HSA binds its ligands in a cleft, *i.e.* binding occurs within rather than on the protein surface [28]. Hence the degree of ionization should not appreciably affect the intercalation of a primary amine in the protein, given that the cationic group is located away from the hydrophobic region in the molecule. In contrast, tertiary amines such as nicardipine have the protonated function at the center of the hydrophobic regions, and their intercalation in the protein appears to be affected by the ionization degree, similarly to the effect observed in octan-1-ol partitioning. Although this model remains speculative and needs more extensive investigations, it is offered as a working hypothesis on the mechanisms of interaction between HSA and ionizable compounds.

AGP Chromatography. All neutral DHPs (except the achiral nifedipine) were enantiomerically resolved with an eluent of propan-2-ol/0.01M phosphate-buffered saline (PBS) pH 7.0 10 : 90 (*v*/*v*), showing two chromatographic peaks with a resolution factor > 50%, with isradipine and nisoldipine resolved at the baseline (*Tables 2* and *3*). Although the results were obtained for racemic mixtures, the behavior of isolated enantiomers, whenever available, showed an absence of interaction between enantiomers.

Table 2. *Enantioselectivity* (α Values) *of an AGP Column for Chiral Neutral DHPs at Different Concentrations of DMOA in the Eluent*[a])

	[DMOA]/mM				
	0	1.00	2.00	3.00	4.00
Nitrendipine	1.34	1.25	1.20	1.10	1.00
Nimodipine	1.32	1.22	1.15	1.00	1.00
Isradipine	2.34	1.69	1.52	1.34	1.10
Felodipine	1.32	1.31	1.18	1.00	1.00
Nisoldipine	2.30	1.37	1.19	1.00	1.00

[a]) Eluent: propan-2-ol/0.01M phosphate-buffered saline (PBS) pH 7.0 10 : 90 (*v*/*v*); flow rate 0.9 ml/min. $\alpha = k'_R/k'_S$.

Because amlodipine was eluted as an extremely broad peak and nicardipine was retained on the column, it was not possible to use the above eluent for the two basic DHPs. Therefore, their k' values at 10% propan-2-ol were extrapolated from measure-

Table 3. k′ *Values for Neutral DHPs on an AGP Stationary Phase at Different Concentrations of DMOA in the Eluent*[a])

	[DMOA]/mM				
	0	1.00	2.00	3.00	4.00
Nifedipine	2.87 (±0.01)	2.61 (±0.02)	2.60 (±0.02)	2.77 (±0.02)	2.99 (±0.02)
(+)-(*R*)-Nitrendipine	7.72 (±0.02)	7.07 (±0.02)	6.89 (±0.02)	6.69 (±0.03)	7.04 (±0.03)
(−)-(*S*)-Nitrendipine	5.76 (±0.02)	5.64 (±0.02)	5.72 (±0.02)	6.06 (±0.02)	7.04 (±0.02)
(+)-(*R*)-Nimodipine	8.95 (±0.02)	6.96 (±0.03)	6.31 (±0.03)	5.95 (±0.03)	6.27 (±0.03)
(−)-(*S*)-Nimodipine	6.79 (±0.02)	5.72 (±0.02)	5.47 (±0.03)	5.95 (±0.03)	6.27 (±0.03)
(−)-(*R*)-Isradipine	16.38 (±0.02)	11.11 (±0.03)	10.03 (±0.02)	8.68 (±0.02)	8.89 (±0.02)
(+)-(*S*)-Isradipine	7.00 (±0.02)	6.59 (±0.01)	6.62 (±0.02)	6.47 (±0.02)	7.63 (±0.02)
(+)-(*R*)-Felodipine	24.07 (±0.03)	22.47 (±0.03)	21.87 (±0.03)	20.02 (±0.03)	21.53 (±0.04)
(−)-(*S*)-Felodipine	18.22 (±0.02)	17.10 (±0.03)	18.61 (±0.03)	20.02 (±0.03)	21.53 (±0.04)
(−)-(*R*)-Nisoldipine	41.27 (±0.04)	29.53 (±0.04)	27.41 (±0.04)	23.20 (±0.04)	25.22 (±0.04)
(+)-(*S*)-Nisoldipine	17.93 (±0.03)	21.54 (±0.03)	23.00 (±0.04)	23.20 (±0.04)	25.22 (±0.04)

[a]) Eluent: propan-2-ol/0.01M phosphate-buffered saline (PBS) pH 7.0 10 : 90 (*v*/*v*); flow rate 0.9 ml/min; $n \geq 3$.

ments performed at 15, 17, and 20% of propan-2-ol. Under these conditions, however, no enantiomer resolution was observed for the two basic DHPs.

The resolution observed on AGP for neutral DHPs points to enantioselective sites interacting by a mechanism different from that previously demonstrated for bases and involving ion exchange and/or ion pairing [29]. To elucidate the mechanism of retention of the (*R*)- and (*S*)-forms, and to understand why the basic DHPs were not resolved, we investigated the relationship between log *k*′ and lipophilic parameters. The existence of a relationship only for the less-retained isomers ((*S*)-forms) would indicate that the interaction between the (*R*)-forms and the enantioselective site involves retention forces different from lipophilicity.

The relation between retention on AGP and lipophilic parameters of neutral DHPs (including nifedipine) was described by *Eqns. 4–7*. For the (*S*)-forms:

$$\log k'_S = 0.676\ (\pm 0.120) \cdot \log k_w^{IAM} - 0.684\ (\pm 0.287) \quad n = 6\ r = 0.942\ s = 0.115 \tag{4}$$

$$\log k'_S = 0.533\ (\pm 0.102) \cdot \log P - 1.321\ (\pm 0.427) \quad n = 6\ r = 0.934\ s = 0.122 \tag{5}$$

and for the (*R*)-forms:

$$\log k'_R = 0.766\ (\pm 0.283) \cdot \log k_w^{IAM} - 0.712\ (\pm 0.673) \quad n = 6\ r = 0.804\ s = 0.268 \tag{6}$$

$$\log k'_R = 0.672\ (\pm 0.173) \cdot \log P - 1.721\ (\pm 0.728) \quad n = 6\ r = 0.889\ s = 0.207 \tag{7}$$

As can be seen, only the retention of the (*S*)-forms is adequately accounted for by log *P* and log k_w^{IAM}, the latter being slightly better. This indicates that the stereoselective component of the retention of the (*R*)-forms is not related to lipophilicity. Moreover, the retention on AGP of nifedipine, the non-chiral DHP, was predicted adequately only by the relationship between log k_s' and log k_w^{IAM} obtained on the basis of five data points relative to the five chiral neutral DHPs, whereas the other equations interrelating log k' values and lipophilicity on the basis of five data points mispredicted the interaction of nifedipine on AGP (equations not shown). Therefore, nifedipine showed an '(*S*)-form-like' behavior, since no retention interactions like for the (*R*)-forms was observed.

In the case of nicardipine and amlodipine, it cannot be excluded that their retention mechanism on AGP differed from that of neutral DHPs. Indeed, the analytical conditions were different (see above), and the compounds, being extensively ionized, were able to interact electrostatically with the protein. To clarify the role played by lipophilicity in the retention of nicardipine and amlodipine, we considered only *Eqns. 4* and *5*, given that the relationships for the (*R*)-forms were less significant.

The inclusion of nicardipine (log $k' = 1.664$) in *Eqn. 4* does not affect the quality of the correlation (*Eqn. 8*), whereas the inclusion in *Eqn. 5* yields a slightly less significant relation (*Eqn. 9*).

$$\log k_s' = 0.782\ (\pm 0.106) \cdot \log k_w^{IAM} - 0.914\ (\pm 0.264) \quad (8)$$
$$n = 7\ r = 0.957\ s = 0.126$$

$$\log k_s' = 0.650\ (\pm 0.116) \cdot \log P - 1.777\ (\pm 0.504) \quad (9)$$
$$n = 7\ r = 0.928\ s = 0.163$$

More interesting is the case of amlodipine because it has a much higher log k_w^{IAM} than expected on the basis of its log *P* in comparison to the neutral DHPs. Its log k' on AGP (1.506) is strongly underestimated, not only on the basis of its log *P* value (log k' predicted by *Eqn. 5* is 0.438), but also on the basis of its log k_w^{IAM} value (log k' predicted by *Eqn. 4* is 1.066). Therefore, the inclusion of amlodipine worsened *Eqn. 8* relative to the relationship log k_w^{IAM}/log k_s' (*Eqn. 10*), whereas for *Eqn. 9* (relative to the relationship log *P*/log k_s') any significance was lost.

$$\log k_s' = 0.813\ (\pm 0.157)\ \log k_w^{IAM} - 0.940\ (\pm 0.395) \quad (10)$$
$$n = 8\ r = 0.904\ s = 0.189$$

It is important to remember that the log k_w^{IAM} value of amlodipine was shown to take into account not only lipophilic but also electrostatic interactions with phospholipids. However, that this parameter was not able to correctly estimate the interactions on AGP indicates that its electrostatic component is quantitatively different from that with phospholipids.

It can, therefore, be concluded that only the (*S*)-form of neutral congeners has a mainly lipophilicity-based interaction with AGP, whereas basic compounds and the (*R*)-forms interact by a more complex mechanism not adequately accounted for by either log *P* or log k_w^{IAM}. However, the log k_w^{IAM} parameter proved better than log *P* to describe the retention of the neutral congeners. Because the retention of neutral DHPs

on both HSA and AGP (but only for the (*S*)-forms) strongly depends on lipophilicity, the interaction values on the two proteins are strongly interrelated as shown by *Eqn. 11*.

$$\log k'_S = 1.211\ (\pm 0.193)\ \log k'_{HSA} - 0.180\ (\pm 0.178) \qquad (11)$$
$$n = 6\ r = 0.952\ s = 0.105$$

High Performance Displacement Chromatography on AGP. Dimethyloctylamine (DMOA) was used as a displacer since it is known to affect both the retention and resolution of various analytes [17][30][31]. The way in which the k' value of a solute is affected by the displacer yields information on the binding interactions that may exist between the two compounds. Moreover, the different sensitivity of two enantiomers can clarify their binding capability to specific enantioselective sites on the protein.

A mathematical model has been developed to describe the competition between two solutes for binding to one site [17][31][32]. When simple competition occurs at one site, a linear relationship should be found between $1/k'_A$ and the concentration of the displacer [D] according to *Eqn. 12*, where k'_A is the ratio between n_S (= number of mol of analyte in the stationary phase) and n_M (= number of mol of analyte in the mobile phase) and is directly proportional to the chromatographic capacity factor, k'; V_M is the volume of mobile phase; S is the surface area of the stationary phase; K_{AP} and K_{DP} are the affinity constants of analyte and displacer, respectively; and $[P]_T$ is the concentration of the binding site on the stationary phase. Furthermore, a second model describing competition at one site with binding at secondary site(s) unaffected by DMOA was developed (see *Eqn. 13*), where X represents the binding at the sites unaffected by the competitor. Therefore, the observation of a linear relationship upon plotting either $1/k'$ or $1/(k' - X)$ *vs.* [D] is indicative of a competition between the analyte and the displacer to bind to the same site. Moreover, the affinity constant of displacer, K_{DP}, can be easily calculated (K_{DP} = slope/intercept).

$$1/k'_A = V_M/(S \cdot K_{AP} \cdot [P]_T) + V_M/(S \cdot K_{AP} \cdot [P]_T) \cdot K_{DP} \cdot [D] \qquad (12)$$

$$1/(k'_A - X) = V_M/(S \cdot K_{AP} \cdot [P]_T) + V_M/(S \cdot K_{AP} \cdot [P]_T) \cdot K_{DP} \cdot [D] \qquad (13)$$

The values of k' at different concentrations of DMOA for nifedipine, nitrendipine, nimodipine, nisoldipine, felodipine, and isradipine are reported in *Table 3*. As can be seen, all (*R*)-isomers eluted faster at higher concentrations of the displacer. The addition of 1 mM DMOA to the mobile phase resulted in a decrease in their k' values: 10% for nitrendipine, 23.5% for nimodipine, 32.1% for isradipine, 6.6% for felodipine, and 28.4% for nisoldipine. Additional decreases in their k' values were observed up to 3 mM DMOA. At 4 mM DMOA, the k' values were slightly higher than at 3 mM.

The effect of DMOA on k' values of the (*S*)-forms was negligible, with the exception of (+)-(*S*)-nisoldipine which showed increasing k' values on addition of DMOA. Moreover, analogously to the (*R*)-forms, the k' values of the (*S*)-forms at 4 mM DMOA were slightly higher than at 3 mM.

The decrease in retention of the (*R*)-forms and the simultaneous constancy of retention of the (*S*)-forms (increase for nisoldipine) when adding DMOA resulted in a progressive reduction of stereoselectivity (*Table 2*). A complete loss of stereoselectiv-

ity was observed at either 3 mM DMOA (for nimodipine, felodipine, and nisoldipine) or 4 mM DMOA (for nitrendipine). For isradipine, a complete loss of stereoselectivity was not observed up to 4 mM DMOA.

These results indicate that DHPs bind to at least two different sites on the protein. First, there appear to be enantioselective sites that bind only the (*R*)-forms and are negatively affected by the addition of DMOA to the eluent. Second, there are non-enantioselective sites that account for the increase in retention at higher DMOA concentrations for both the (*R*)- and (*S*)-forms, probably due to allosteric interactions.

The enantioselective sites were more sensitive to DMOA than the non-enantioselective ones. Indeed, we observed that the increase in retention (probably due to cooperative interactions) only became evident at DMOA concentrations that abolished (probably by competitive interactions) the enantioselective properties of the protein, indicating a complete saturation of the enantioselective sites.

Although the reduced retention of the (*R*)-DHPs is believed to arise from a competitive displacement of DMOA, this hypothesis could not be demonstrated. In fact, models of single competition at one site did not describe the effect of DMOA on the (*R*)-DHPs. This is probably due to the difficulty in estimating the *X* value to be introduced in *Eqn. 13*, since the retention of the (*S*)-forms is controlled at least partly by binding to DMOA-sensitive sites, thus the k' values obtained for the (*S*)-forms in the absence of DMOA are not reliable. Moreover, the workable range of DMOA concentrations was too narrow and insufficient to obtain a significant relationship, because retention data at 4 mM DMOA were unsuitable, being affected by the cooperative effect of the displacer on the non-enantioselective sites.

Nifedipine, the non-chiral DHP considered in this study, behaved as the (*S*)-forms, indicating that it did not bind to the enantioselective sites. It is interesting to note that this conclusion is in line with the relation between retention and lipophilicity seen for nifedipine.

Conclusion. – DHPs are a unique class of structurally related drugs that are comprized of both neutral and ionizable compounds. This makes the values of lipophilicity expressed by log *P* non-collinear to those expressed by $\log k_w^{IAM}$ and renders both parameters *a priori* interesting for the prediction of serum-protein binding.

Interactions of HSA are so strongly governed by lipophilicity that no stereoselective mechanism could be shown, with the exception of isradipine. The octan-1-ol/H_2O system was more effective than the IAM column in yielding data describing HSA interactions with basic DHPs. However, it is not clear in which cases it would be beneficial to correct log *P* for ionization.

The presence on AGP of enantioselective binding sites for (*R*)-DHPs was demonstrated. Only the retention of (*S*)-DHPs on AGP was related to lipophilicity. DMOA can bind to the enantioselective sites, producing a progressive loss of stereoselectivity of the protein. However, an increase in retention was observed for both (*R*)- and (*S*)-forms at higher DMOA concentrations. This indicates that at least two different binding sites for DHPs are present on AGP, a family of enantioselective ones having high affinity for DMOA, and a family of non-enantioselective ones with low affinity for DMOA. These two families of sites are probably affected by DMOA by a competitive and cooperative mechanism, respectively.

Lipophilicity parameters obtained with IAM columns were insufficient to describe the interactions between basic, ionized DHPs and serum proteins. In contrast, these parameters were more effective than log *P* in the case of neutral DHPs. This could indicate that compared to octan-1-ol, an amphipathic phase such as phospholipids can better mimic the partitioning of neutral compounds into the proteins.

Finally, we note that the presence of specific binding sites for DHPs on AGP could play a role in the *in vivo* occurrence of drug-drug interactions. The ability of AGP columns to demonstrate such interactions should be explored.

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