CHIRALITY 7:23–27 (1995)

Retention Model for the Resolved Enantiomers of Felodipine on Chiral-AGP Using Micellar Mobile Phases

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ABSTRACT A retention model for the chiral separation of an uncharged solute, felodipine, on CHIRAL-AGP, using a micellar mobile phase is proposed. The model assumes the presence of two stereoselective sites and each enantiomer was found to interact with different sites. Addition of a chiral aliphatic alcohol, (+)-(S)-2-octanol, preferentially interacted with the binding site for (−)-(S)-felodipine. The monomeric form of the micellar agent (Tween® 20) competed with the enantiomers for the adsorption sites, and the formation of a 1:1 complex between the enantiomers and the micelles was assumed. The retention of the solutes was effectively controlled by adding small quantities ($<1.63 \times 10^{-3}$ *M*) of the nonionic detergent Tween 20 to the mobile phase. Baseline separation was achieved by addition of 1.0 m*M* *n*-octylamine to the mobile phase; 8.14×10^{-4} *M* Tween 20 in phosphate buffer pH 7.0. The separation factor ($\alpha = 1.74$) was unaffected by the detergent concentration in the presence of 1.0 m*M* *n*-octylamine.

KEY WORDS: felodipine, retention model, micellar mobile phases, chiral resolution, CHIRAL-AGP

The enantiomeric resolutions of three hydrophobic amines[1] and an acid[2] using Tween® 20 as an organic modifier along with different charged additives on α_1-acid glycoprotein columns have been described previously. The experiments were typically performed by decreasing the retention time by addition of increasing concentrations of a detergent, and thereafter adding a suitable counterion in order to increase the enantioselectivity. This protocol proved to be particularly useful for the hydrophobic enantiomers studied as it gave short retention times with maintained stereoselectivity. Organic solvents like methanol or acetonitrile have been used extensively as mobile phase modifiers in order to shorten retention times and/or give better peak shape. Unfortunately, the presence of high concentrations of uncharged modifiers usually ruins the stereoselectivity, and thus the usefulness of this approach is limited when dealing with highly retained solutes.

Although the mechanism of chiral recognition by the protein is largely unknown, a retention model study can give a general conception of the main types of solute–micelle–protein interactions involved. In order to simplify the system, an aprotic solute, felodipine, was chosen as a test substance for evaluation of the retention model.

In 1964 Herries et al. developed an equation which described the elution of a small solute on a Sephadex G-25 column with a micellar mobile phase.[3] The derivation outlined closely followed the classical work of Martin and Synge on partition chromatography[4] and also accounted for molecular sieving and micelle partitioning effects. In 1981 Armstrong and Nome introduced the three-phase model for micellar liquid chromatography and also treated the relationship between solute retention and micellar mobile phase composition.[5] Later Arunyanart and Cline Love[6] modelled the retention equation in the form of binding constants and capacity factors rather than partition coefficients and elution volumes. The subject of retention models for micellar systems has been adequately reviewed by Armstrong.[7]

The aim of this investigation was to develop a retention model for an uncharged solute, felodipine, in a micellar liquid chromatographic system. The effects of different aliphatic amines as well as the concentration of Tween 20 on enantiomeric resolution and retention are reported.

MATERIALS AND METHODS

Chemicals

(+)-(R)- and (−)-(S)-3,5-Pyridinedicarboxylic acid, 4-(2,3-dichlorophenyl)-1,4-dihydro-2,6-dimethyl, 5-ethyl-3-methyl ester (felodipine) were gifts from Astra Hässle AB (Sweden) Tween 20, (+)-(S)-2-octanol (ChiraSelect) and triethylamine (puriss p.a.) were purchased from Fluka Chemie AG (Buchs, Switzerland). Ortho-phosphoric acid (p.a.) was from E. Merck (Darmstadt, Germany). *N,N*-dimethyloctylamine (DMOA) was purchased from Aldrich-Chemie (Steinheim, West-Germany), and octylamine-HCl was from Eastman Organic Chemicals (Rochester, NY, USA). Heptylamine

Received for publication May 25, 1994; accepted August 26, 1994.
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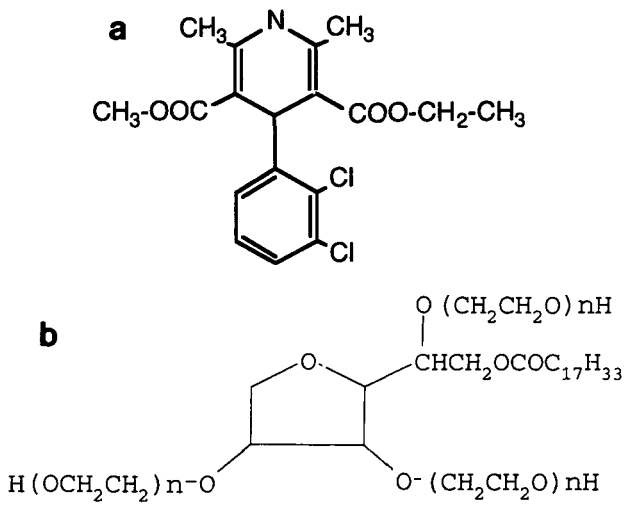

Fig. 1. (a) Felodipine; (b) Tween 20.

(99+%) was purchased from Janssen Chimica (2340 Beerse, Belgium). Sodium hydroxide was from EKA Nobel (Sweden).

Apparatus

The chromatographic system consisted of two LKB 2150 HPLC pumps (Pharmacia, Uppsala, Sweden), a LKB 2152 HPLC Controller, a LKB 2152-400 high pressure mixer, and a Kontron sampler MSI 660 controller (Kontron Electrolab, London, England). The UV absorbance was monitored at either 250 or 280 nm using a Waters lambda max model 480 LC spectrophotometer or a Waters lambda max model 481 LC spectrophotometer (Waters Associates, Milford, MA, USA). A Perkin-Elmer LS-4 fluorescence spectrometer (Norwalk, CT, USA) was also used at excitation wavelength 363 nm and emission wavelength 422 nm. The recorder was a Kipp&Zonen BD 40 (Kipp&Zonen, Delft, The Netherlands). The separations were performed on different CHIRAL-AGP columns (100 × 4.0 mm) from Chrom Tech AB (Norsborg, Sweden). The column, the mobile phase, and the interfacial tensiometer (mentioned below) were thermostatted with a HETO Type 02 PT 923 TC waterbath, (Birkerød, Denmark). The pH measurements were made with an AG 9100 Metrohm 632 pH meter (Herisau, Switzerland) equipped with a Type 1014 glass pH electrode.

Chromatographic Conditions

The standard conditions for chromatography were a flow rate of 0.90 ml/min and a column and mobile phase temperature of 45°C. The mobile phases were prepared by making a phosphate buffer (I = 0.1) from 1 M phosphoric acid and 1 M sodium hydroxide to which different modifiers were added. The pH was then adjusted (when necessary) to the desired level by adding a few drops of 1 M phosphoric acid or 1 M sodium hydroxide. The influence of this addition on the ionic strength was negligible as the amount added was very small.

Critical Micelle Concentration (CMC) Determination

The equipment used for the CMC determination was a KRÜSS interfacial-tensiometer K 8600 E/E (Hamburg, Germany) thermostated to 45°C. The surface tension was measured by the du Noüy ring detachment method.[8] The solutions used for the CMC determination were prepared as follows: a proper amount of Tween 20 was added to a mixture of 1.0 mM n-octylamine in phosphate buffer pH 7.0; pH was then measured and if necessary adjusted to pH 7.0. The CMC was evaluated by the intersection point obtained by linear regression of the values obtained in the surface measurement. The CMC value of the solutions was found to be 3.42×10^{-4} M assuming a molecular weight of 1228 for Tween 20.[9]

TABLE 1. Influence of organic modifier on chiral resolution[a]

Additive	k'_R	α^b	R_S^c
—	6.50	1.34	1.00
1.0 mM trietylamine	6.82	1.35	1.02
1.0 mM heptylamine	5.84	1.50	1.38
1.0 mM octylamine	6.27	1.70	1.81
1.0 mM N,N-dimethyloctylamine	5.36	1.43	1.22

[a]Separation column, CHIRAL-AGP, 100 × 4.0 mm; mobile phase, phosphate buffer pH 7.0 and 8.14×10^{-4} M Tween 20 (I = 0.1); solute, racemate felodipine (0.54 nmol); temperature, 45°C.
[b]$\alpha = k'_S/k'_R$.
[c]$R_S = 2(t_{R2} - t_{R1})/(W_{t2} + W_{t1})$.

Evaluation of Retention Models

To evaluate the retention model PCNONLIN version 3.0 (SCI Software, Lexington, KY, USA) was used. The method chosen for the estimation of constants and confidence limits was the Levenberg-type modification of the Gauss–Newton algorithm.[10]

RESULTS AND DISCUSSION

Effects of Modifiers

The enantioselective retention of solutes on a protein based phase (e.g., albumin, α_1-acid glycoprotein and cellulase) are generally optimized by varying pH, ionic strength, and/or by adding either charged or uncharged modifiers.[11–13] A few retention models have been proposed to explain the effect of the mobile phase composition on the stereoselective retention to the α_1-acid glycoprotein phase.[14,15] The enantiomers were then assumed to be distributed in charged or uncharged form to one or two adsorption sites on the protein molecule.

Previously, we examined micellar mobile phases as an alternative to common organic modifiers for regulating the enantioseparation of hydrophobic enantiomers (naproxen, propranolol, remoxipride, trimipramine) on Enantiopac and CHIRAL-AGP.[1,2] The results obtained indicate several interesting properties of the micellar systems used, including reduced retention times with a maintained enantioselectivity for the hydrophobic enantiomers.

In order to simplify the evaluation of the retention model in micellar chromatography and protein stationary phases an aprotic hydrophobic solute, felodipine (structure in Fig. 1) was chosen as the model substance. A mobile phase composed of 8.14×10^{-4} M Tween 20 in phosphate buffer pH 7.0 resulted

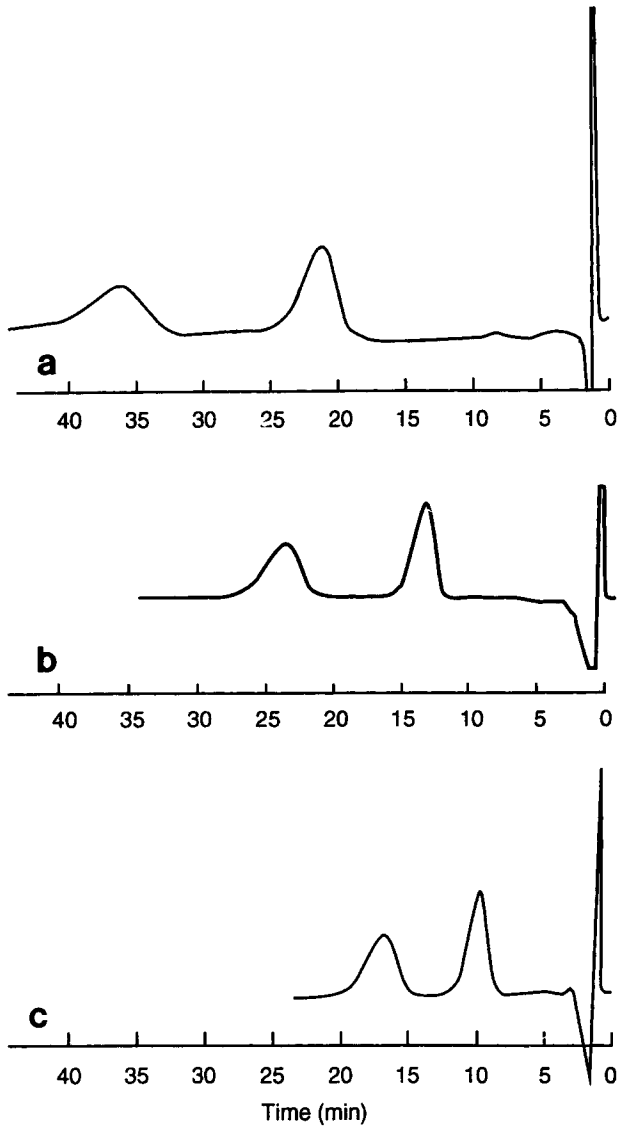

Fig. 2. Separation of (R/S)-felodipine. Mobile phase: 1.00 m*M* octylamine in phosphate buffer pH 7.0 and (**a**) 2.85×10^{-4} *M* Tween 20, (**b**) 4.7×10^{-4} *M* Tween 20 and (**c**) 6.11×10^{-4} *M* Tween 20. Separation column, CHIRAL-AGP, 100 × 4.0 mm. Flow rate, 0.90 ml/min. Temperature, 45°C

in incomplete resolution of the felodipine enantiomers (Table 1). As discussed above the use of charged additives to control the enantioselectivity and retention on CHIRAL-AGP is well established in the literature.[12] The effect of the modifiers is highly dependent on their structure and is difficult to predict (see Table 1). Addition of triethylamine increased the retention for both enantiomers, while only slightly affecting the separation factor. The more hydrophobic heptylamine (seven carbons) decreased the retention for the first eluted enantiomer (R) while the second was almost unaffected. The complexity of the chiral recognition is further illustrated by the fact that addition of 1.0 m*M* octylamine (eight carbons) only slightly affected retention of the (R)-enantiomer while the retention of the (S)-enantiomer was strongly increased. A

TABLE 2. Influence of Tween 20 concentration on chiral resolution[a]

Tween 20 (*M*)	k'_R	α
0–6.51×10^{-5}	n.d.	n.d.[b]
8.14×10^{-5}	48.8	1.71
1.63×10^{-4}	37.2	1.76
1.79×10^{-4}	34.9	1.74
2.44×10^{-4}	25.3	1.82
2.85×10^{-4}	24.7	1.73
3.26×10^{-4}	17.8	1.74
4.07×10^{-4}	15.4	1.77
4.89×10^{-4}	10.8	1.70
6.11×10^{-4}	10.3	1.79
8.14×10^{-4}	6.70	1.76
9.77×10^{-4}	5.08	1.69
1.63×10^{-3}	3.04	1.68

[a]Separation column, CHIRAL-AGP No. 2, 100 × 4.0 mm; mobile phase, Tween 20 in phosphate buffer pH 7.0 and 1.0 m*M* octylamine ($I = 0.1$); solute, (R/S)-felodipine (1.1–5.5 nmol); temperature, 45°C.
[b]n.d., not detected.

TABLE 3. Estimation of constants evaluated

Parameter	Estimate	95% Conf. limits
(R)-Felodipine		
$q \times K \times K°$	88.0	75.3–100
K_T	9880	7460–12300
K_M	4400	3260–5540
(S)-Felodipine		
$q \times K \times K°$	144	124–165
K_T	8890	6680–11100
K_M	4650	3420–5880

drastic improvement of the separation factor was observed. It has been proposed that this kind of effect can be explained by a conformational change of the protein.[16] Exchange of octylamine for *N,N*-dimethyloctylamine (DMOA) decreased the chiral separation. Thus, octylamine is preferred as it gives complete resolution of (R,S)-felodpine (Fig. 2).

Retention Model

It is assumed that the enantiomers of felodipine and Tween 20 as a monomer are adsorbed to one site, A_s, on the protein molecule [Eqs. (1) and (2), respectively]:

$$F_{aq} + A_s = FA_S K = [FA]_s/([F]_{aq} \times [A]_S) \quad (1)$$

$$T_{aq} + A_S = TA_S K_T = [TA]_S/([T]_{aq} \times [A]_S) \quad (2)$$

Where FA_S is the total concentration of sample (F) adsorbed to the site and TA_S is the concentration of Tween monomer (T) adsorbed, both expressed in moles/g. The monolayer adsorption capacity of the protein molecule, $K°$, is limited and is expressed by Eq. (3) (cf. reference 17):

$$K° = [A]_S + [FA]_S + [TA]_S \quad (3)$$

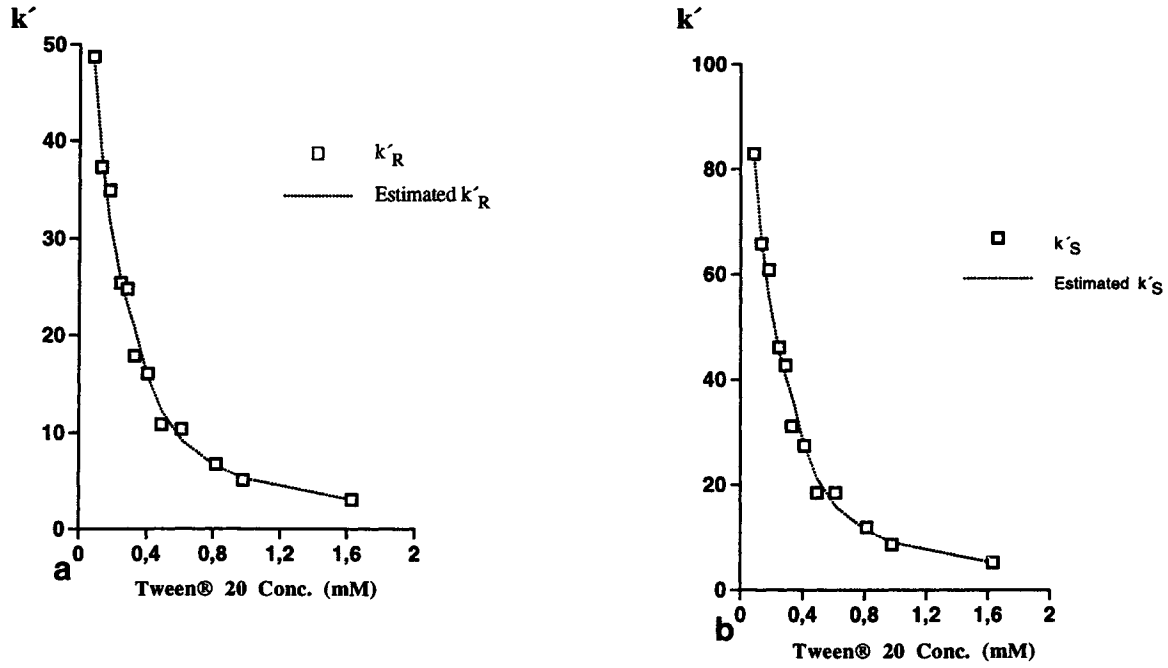

Fig. 3. (a) Observed and estimated k'_R values. (b) Observed and estimated k'_S values. Mobile phase: Tween 20 and 1.0 m*M* octylamine in phosphate buffer pH 7.0.

TABLE 4. Effect of (+)-(S)-2-octanol concentration on chiral resolution[a]

Concentration (m*M*)	k'_S	k'_R	α^b
0	22.3	14.8	1.51
1.0	19.4	15.5	1.25
5.0	12.3	13.6	0.90

[a]Separation column, CHIRAL-AGP No. 2, 100 × 4.0 mm; mobile phase, phosphate buffer pH 6.0 and $3.26 \times 10^{-4}M$ Tween 20 ($I = 0.1$); solute, (R/S)-felodipine (1.1–5.5 nmol); temperature, 25°C.
[b]$\alpha = k'_S/k'_R$.

The distribution of the felodipine enantiomers to the micelles, M, in the mobile phase is expressed by Eq. (4):

$$F_{aq} + M = FM_{aq} \quad K_M = [FM]_{aq}/([F]_{aq} \times [M]) \quad (4)$$

where M = (detergent concentration − CMC). The aggregation number for the micelles of Tween 20 is incorporated in the K_M value. A combination of Eqs. (1)–(4) gives the following expression for the capacity factor of the sample F assuming that $K \times F_{aq} << (1 + K_T \times T_{aq})$, i.e., the retention is independent of the concentration of the solute:

$$K' = \frac{q \times K \times K^0}{(1 + K_M \times [M])\ (1 + K_T \times [T]_{aq})} \quad (5)$$

where q is the ratio of amount of adsorbent to volume of mobile phase in the chromatographic system expressed in g/liter. Distribution of the competing amine to the protein molecule, i.e., occupation of stereoselective sites available for the enantiomers, is regarded to have constant influence on the adsorption of felodipine enantiomers in order to simplify the equations. The capacity and separation factors at different concentrations of Tween 20 is given in Table 2. The drastic decrease in retention of felodipine enantiomers in the presence of Tween 20 in concentrations below the CMC (3.42×10^{-4} *M*) suggests a strong competitive effect of the monomer to the same adsorption site(s) as the enantiomers of felodipine. The separation factors were plotted on normal probability paper in order to verify normal distribution. As the plot gave a fairly straight line, the conclusion was that the separation factor could be considered constant in the region studied.

In order to estimate the constants in Eq. (5) nonlinear regression was performed. The results of the evaluation are presented in Table 3 and Figure 3. No significant difference was obtained for K_M, i.e., no enantioselective distribution to the micelles occurred despite the fact that Tween 20 has several chiral carbons (Fig. 1).

No significant difference in the adsorption of the Tween monomers (K_T) was obtained. In practice, however, they may have different values, since evidence shows (see discussion below) that two different enantioselective binding sites are operative. The affinity of the monomers may be different at the two sites. It was not possible to evaluate the two constants, K_M and K_T, by separate regression analysis, since the monomer competition had a significant impact on the k' values also at higher Tween concentrations (the micellar region).

The enantioselectivity is generally correlated to the ratio of binding constants (K) for the enantiomers to the chiral selector. However, the difference in binding affinity for the two enantiomers observed does not necessarily mean that the binding constants K differ for the two enantiomers as the

adsorption capacity ($K°$) for two separate binding sites for the enantiomers may differ.

Results using a chiral alcohol, (+)-(S)-2-octanol, as mobile phase additive support the model of two independent stereoselective binding sites on the immobilized protein (Table 4). Increasing concentration of the chiral alcohol in the presence of Tween 20 showed a preferential interaction of (+)-(S)-2-octanol with the binding site for (S)-felodipine giving rise to a reversal of the elution order of the enantiomers at the highest (+)-(S)-2-octanol concentration. The retention of the (R)-enantiomer was only slightly affected. An alternative explanation for this reversal in elution order of the enantiomers may be allosteric interaction as discussed by Wainer and Noctor.[18]

In conclusion, this study indicates the existence of two different binding sites on α_1-acid glycoprotein for felodipine. A retention model based on this assumption has been developed. For proteolytic solutes or solutes having another number of available sites the model has to be modified taking in consideration protolytic functions and number of sites. The retention of the enantiomers can be regulated by the Tween concentration with chiral resolution maintained. Furthermore, the retention order of the enantiomers can be regulated by addition of a modifier to the mobile phase which interacts preferably with one of the binding sites on the protein molecule.

ACKNOWLEDGMENTS

The support of this work by the Swedish National Science Council is gratefully acknowledged. Research grants from Swedish Academy of Pharmaceutical Sciences and the I.F. Foundation for Pharmaceutical Research are gratefully acknowledged.

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