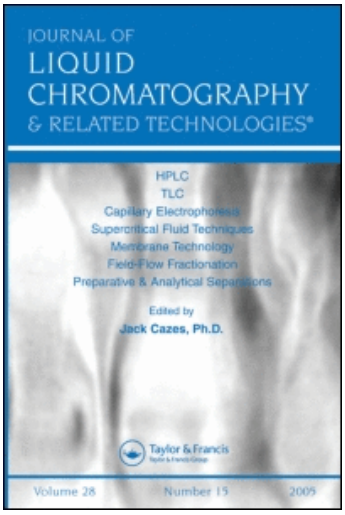

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R. Thompson[a]; V. Prasad[a]; N. Grinberg[a]; D. Ellison[a]; J. Wyvratt[a]
[a] Merck Research Laboratories, Rahway, NJ, U.S.A.

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MECHANISTIC ASPECTS OF THE STEREOSPECIFIC INTERACTIONS OF IMMOBILIZED α_1-ACID GLYCOPROTEIN

R. Thompson,* V. Prasad, N. Grinberg, D. Ellison, and J. Wyvratt

Merck Research Laboratories, P.O. Box 2000,
Rahway, NJ 07065, USA

ABSTRACT

The stereospecific interaction of a neutral probe molecule, acetonide, with immobilized α_1-acid glycoprotein (AGP) was investigated. Enantioselectivity was found to be influenced by the choice of organic modifier, temperature, and pH. These parameters could be varied to the extent that a reversal of elution order could be induced. Our studies found that the role of hydrogen bonding in the chiral discrimination of acetonide was minimal. An inclusion mechanism is proposed with the investigated parameters affecting the access to the binding sites either through induced conformational changes or steric hindrance.

INTRODUCTION

Proteins play a major physiological role in their ability to reversibly bind small molecules such as drugs. The vast number of functional groups possessed

*Corresponding author.

by the proteins allows them to undergo interactions, such as hydrophobic or electrostatic ones, with a broad range of substances. The inherent chirality of proteins allows for stereoselective interactions with chiral compounds (1–3). Protein-based stationary phases have been developed for the separation of enantiomers because of this stereospecific molecular recognition. The identification of the specific sites on the protein that determine chiral discrimination is rendered difficult by the plethora of potential sites of interaction and the uncertainty of their positions on a three-dimensional level.

A protein molecule is a biopolymer possessing a unique folded structure in solution. Its tertiary structure is derived from the combination of secondary structures (such as helices, reverse turns, and pleated sheets) and nonordered segments. The tertiary structure is compact with some spaces intended for access of solvent or for interaction with other molecules. In a polar medium such as water, the tertiary structure is such that the surface is polar and the interior is nonpolar, resulting in a stabilized conformation that places the hydrophilic residues at the surface and buries the hydrophobic residues. The tertiary structure of proteins is thought to be important for stereospecific interactions. It is known that the tertiary structure of proteins can be reversibly affected as a consequence of a change in the properties of its solution media through the addition of modifiers, such as acetonitrile or isopropanol (4–8). Such changes should also induce a change in the chiral selectivity of the protein and, thus, can be used to optimize chromatographic separations of enantiomers.

In this study, we investigated the effect of organic modifiers and inorganic modifiers on the chiral selectivity of a chiral α_1-acid glycoprotein (AGP) column. This protein is a globular glycoprotein containing a 181 amino acid residue polypeptide and 5 carbohydrate chains (9). It is thought to be a major plasma-binding protein for many neutral and basic drugs (10–12). Drugs appear to predominantly bind through hydrophobic interactions (13,14), and there is evidence for electrostatic (15), van der Waals (16), and hydrogen-bonding (17) interactions as well.

The major interaction site is an apolar cavity, which is formed from a folding of a segment containing α-helices, β-sheets, and β-reverse turns (18–23). The binding can be stereoselective (24–27). Conformational changes have been observed in AGP as a function of temperature (7,27–32), mobile phase pH, and mobile phase modifier type (33–35). Such conformational changes should affect binding and specifically stereospecific interactions.

AGP has been used extensively as a chiral stationary phase (CSP) for high-performance liquid chromatography (HPLC) (36–42). The binding of AGP to the stationary phase is believed to cause only minor conformational changes (42). Resolution has been obtained for acidic, basic, and neutral compounds, suggesting multiple binding mechanisms (36,38,42). Enantioselectivity is believed to be achieved through interaction of the solute with a hydrophobic core as observed for the binding of drugs to native AGP (1). The limited information regarding the

tertiary structure of the bound AGP renders the absence of any specific guidelines as to how a particular solute will undergo stereoselective interactions. General guidelines can be obtained, however, by observation of the interaction of probe molecules and the effect of parameters, such as organic modifier, temperature, and pH, on the enantioselectivity.

The probe molecule used in this study is (3aS-*cis*)-3,3a,8,8a-tetrahydro-2,2-dimethyl-3-(1-oxo-3-phenylpropyl-2*H*-indeno[1,2-d]oxazole (referred to as acetonide) (Fig. 1). Both of its chiral centers are held within the same rigid five-membered ring. Only two optical isomers are observed due to steric restrictions. Acetonide is a neutral molecule that excludes electrostatic interactions as one of the chiral recognition mechanisms. Being neutral, any changes observed in stereoselectivity have to be attributed solely to changes in the stationary phase and not to protolysis of the probe.

Additionally, its interaction within the apolar cavity should be enhanced. The enantioselectivity of AGP toward acetonide was investigated as a function of the type of organic modifier, temperature, and pH. All of these parameters are anticipated to have a lesser influence on acetonide itself, which is neutral and possesses rigid stereogenic centers, relative to AGP. Changes in enantioselectivity can, consequently, be attributed, in most cases, to changes occurring with AGP.

EXPERIMENTAL

Reagents

Fisher Scientific (Pittsburgh, PA, USA) HPLC grade (Optima) solvents were used. Buffers were prepared using deionized water from a Picotech Hydro ultrapure water system and potassium phosphate from J. T. Baker (Philipsburg, NJ, USA). Acetonide (individual enantiomers) was supplied by Process Research (Merck Research Laboratories, Rahway, NJ, USA).

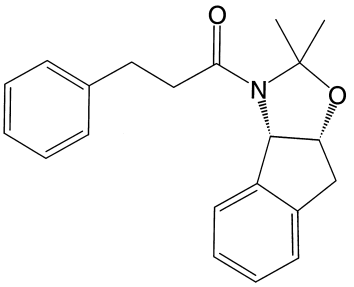

Figure 1. Structure of (−)acetonide.

Chromatographic Equipment

The chromatographic systems used included a P4000 quartenary gradient pump, an AS3000 variable loop autosampler, and a UV1000 single wavelength ultraviolet (UV)-visible programmable detector (Spectra Physics, Piscataway, NJ, USA), and Beckman System Gold Software (San Ramon, CA). The column temperature was controlled with a model 7950 temperature controller (Jones Chromatography, Lakewood, CO, USA). Chromatograms were obtained and processed using Nelson 900 Series Access*Chrom version 1.7 software (Perkin-Elmer, PE, Cupertino, CA). The chiral AGP columns used were obtained from Regis Chemical Co. (Morton Grove, IL, USA). The column was 150 × 4.0 mm and contained 979 mg of AGP attached to a 5-μm silica gel substrate.

Chromatographic Conditions

Except as otherwise noted, all experiments were performed under ambient conditions, with a mobile phase of 10 m*M* potassium phosphate buffer/organic modifier at pH 7.0 at a flow rate of 1.0 mL/min with UV detection at 210 nm. The capacity factor of (−)acetonide is designated as k'_1 throughout this investigation.

RESULTS AND DISCUSSION

Effect of Organic Modifier

The initial investigation into the effect of organic modifier on the enantioselectivity of AGP toward acetonide was a comparative study using acetonitrile and isopropanol as the organic modifier to 10 m*M* potassium phosphate at pH 7. A reversal in the elution order was observed with (−)acetonide eluting first with acetonitrile and second with isopropanol. Such behavior, though rare, is not unprecedented. Reversal of elution order by switching from acetonitrile to isopropanol was reported previously for warfarin with AGP (43).

A similar reversal for an alkaloid was reported using human serum albumin as the CSP (44). The reversal in enantioselectivities was attributed to differences in the hydrogen-bonding properties and the polarity of the modifiers. Isopropanol has strong hydrogen bond (or proton) acceptor and donor groups. Acetonitrile has only weak hydrogen bond acceptor properties.

Two hypotheses have been proposed for these types of reversal of elution orders with protein (44,45). The first is that the modifier is competing with solute enantiomers in binding to discrete groups on AGP possessing different

hydrogen bonding properties, e.g., carbonyl oxygens versus amido hydrogens. With acetonitrile as the modifier, the solute can undergo strong hydrogen bonding interactions with the CSP. With isopropanol as the modifier, the solute's ability to interact through hydrogen bonding with the CSP is significantly reduced through competition with isopropanol.

A second type of interaction, for example, electrostatic or dipole, may then be favored, which may have opposing affinities for the enantiomers that result in a reversal of elution order. This theory assumes interaction sites with opposing enantioselectivities, which is likely given the large number of stereogenic centers present on the CSP. The second theory proposes that the modifiers induce reversible changes in the tertiary structure of the protein in such a way as to reverse the enantioselectivity.

To further the investigation, a comparison of the enantioselectivity of AGP toward acetonide as a function of alcohol modifier was undertaken. The alcohols used were methanol, ethanol, *n*-propanol, and isopropanol. Presumably, since these alcohols possess similar hydrogen-bonding properties one would anticipate smaller changes in enantioselectivity but still be able to determine a trend. In going from methanol to *n*-propanol, a significant decrease in enantioselectivity was observed with a reversal of elution order upon going to isopropanol (Fig. 2). As a comparison, the selectivity for acetonitrile was between that of *n*-propanol and ethanol.

Given that ethanol and isopropanol induce opposing enantioselectivities, it would be anticipated that a ternary system of phosphate buffer (pH 7.0)/ethanol/isopropanol could be adjusted to cancel out the overall competing mechanisms. Such a mixture would result in coelution of the two enantiomers. Ternary mixtures were made up to keep the capacity factor of (+)acetonide constant. Figure 3 shows chromatograms obtained from three such combinations. Mixtures of 11.5% isopropanol/0% ethanol and 0% isopropanol/20% ethanol gave the expected reversal in elution order. A ternary mixture of 6% isopropanol/9.5% ethanol resulted in coeluting peaks, in which the opposing enantioselectivities had effectively canceled each other out.

An additional study was performed in which the total amount of organic modifier was kept constant and the ratio of isopropanol to ethanol was varied. Figure 4 shows a plot of the capacity factor of each enantiomer as a function of the ternary mixture. It can be seen that the capacity factor of (−)acetonide decreases slowly in going from 16% ethanol to 16% isopropanol. For hydrophobic interactions, a decrease in capacity factor is expected, as the mobile phase becomes less polar in going from ethanol to isopropanol. The decrease in the capacity factor of (+)acetonide is much more pronounced, however. At 16% ethanol, the (+)acetonide is more strongly retained, and then as isopropanol is added, there is a large drop in retention compared with (−)acetonide, to the extent that they coelute and then (+)acetonide becomes the less retained species.

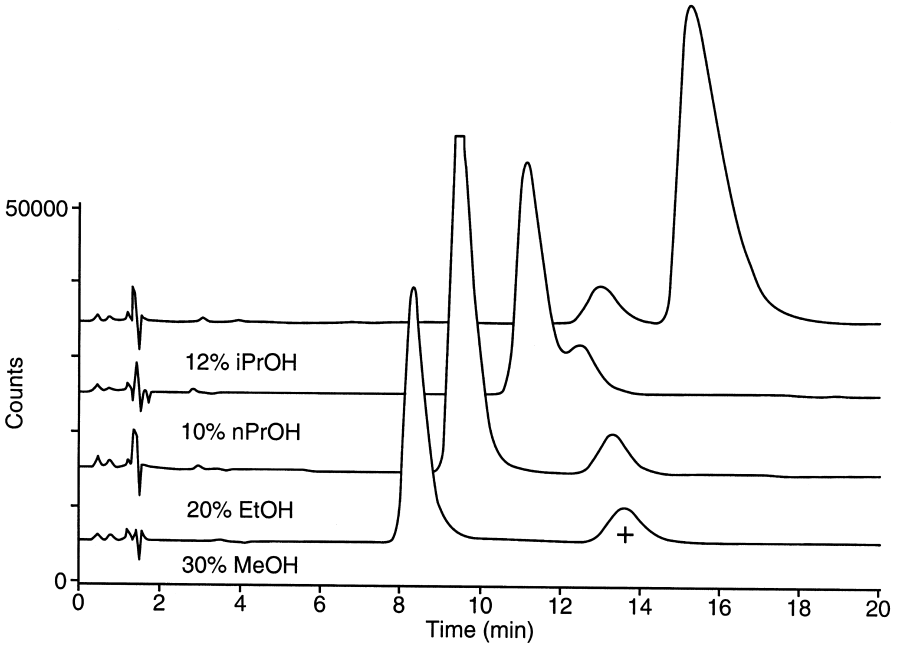

Figure 2. Influence of the type of alcohol modifier on the enantioselectivity. iPrOH, isopropanol; nProH, *n*-propanol; EtoH, ethanol; MeoH, methanol.

The above observations seem to minimize the role of the hydrogen-bonding properties of the modifier in dictating enantioselectivity. Modifiers with similar hydrogen-bonding properties (ethanol versus *n*-propanol) still lead to dramatic changes in enantioselectivity, while modifiers with different hydrogen-bonding properties (ethanol versus acetonitrile) gave similar enantioselectivity. The changes in enantioselectivity seem to be correlated to the bulkiness of the modifier. It is likely that the modifier is inducing changes in the steric environment of the protein. Such changes would vary the accessibility of some sites. This theory is compatible with the possibility that there exist multiple interactive sites, some with opposing enantioselectivity. As access to some of these sites becomes restricted, either due to conformational changes or to steric hindrance by the bulkier alcohol, the enantioselectivity then changes.

Effect of Temperature

To further elucidate the mechanism involved in the binding of acetonide to AGP, temperature studies were performed under different mobile phase composi-

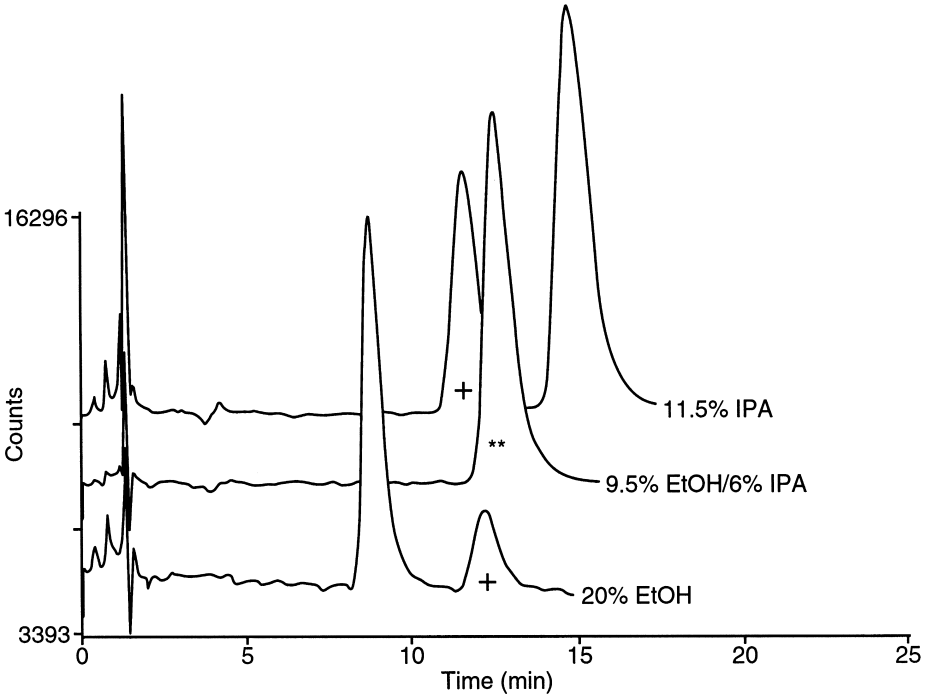

Figure 3. Influence of (ETOH) ethanol/isopropanol (IPA) ratio on the enantioselectivity.

tions of the phosphate buffer (pH 7)/ethanol/isopropanol ternary system. The thermodynamic parameters involved under each mobile phase condition can be determined by using the known relationship between the change in partial molar free energy when the solute is transferred from the mobile phase to the stationary phase (46), This relationship is described in the expression,

$$\ln k' = -(\Delta G^\circ/RT) + \ln \Phi,$$

where Φ represents the phase ratio. The capacity factor is a summation of both achiral and chiral interactions that the solute undergoes with the CSP. The free energy term can be broken into its enthalpic and entropic components to obtain the van't Hoff equation,

$$\ln k' = -(\Delta H^\circ/RT) + \Delta S^\circ/R + \ln \Phi.$$

A plot of ln (capacity factor) against the reciprocal of the absolute temperature should be linear with a slope representing the enthalpic term and an intercept representing the entropic term. Similarly, for enantiomers one can derive a simi-

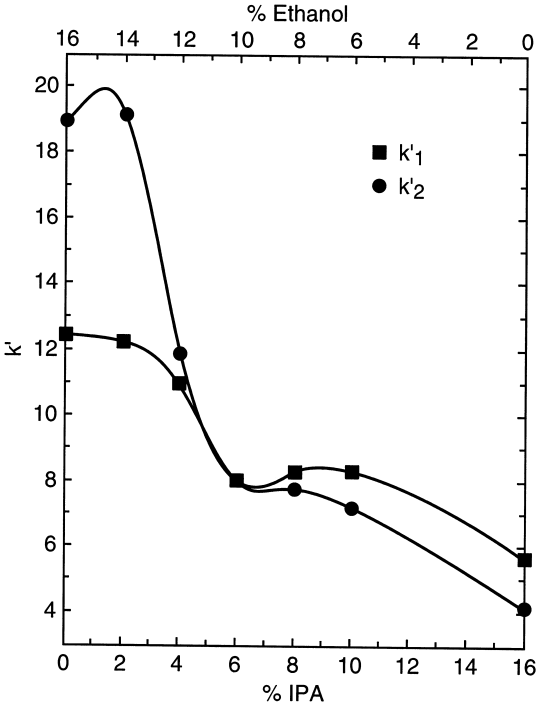

Figure 4. Influence of ethanol/isopropanol ratio on the capacity factors.

lar expression for the selectivity factor (α), which is the ratio of the capacity factors, as a function of temperature,

$$\ln (k'_2/k'_1) = \ln \alpha = -(\Delta\Delta H^\circ/RT) + \Delta\Delta S^\circ/R.$$

Thermodynamic parameters at different compositions of organic mobile phase were obtained from van't Hoff plots. The working temperature range was from −4 to 50°C at 5° intervals. The mobile phase compositions of phosphate buffer (pH 7)/ethanol/isopropanol used were A (80:20:0), B (84.5:9.5:6.0), and C (88.5:0:11.5). These compositions produced the chromatograph shown in Figure 3, representing a coelution and a reversal in elution order. The ternary mobile phases were made up so that the capacity factors obtained for (+)acetonide would be constant within 1 unit.

All of the generated plots for both ln k' and ln α were linear. This linearity indicates that over the temperature range studied and for each of these mobile phases, there appears to be no change in the mechanism involved in the binding

of acetonide to AGP. A comparison of the thermodynamic parameters obtained with the three mobile phases is quite revealing (Tab. 1). With mobile phase A, the enthalpic terms for the individual enantiomers have a magnitude similar to those of the entropic terms. These findings can be interpreted as both enantiomers having similar access to the achiral sites and to the binding site that determines enantioselectivity. (+)Acetonide interacts more strongly with the binding site under these conditions. With mobile phase C, the enthalpic and entropic terms for the enantiomers have diverged greatly in magnitude.

The presence of isopropanol has, either through steric hindrance or inducement of a conformational change in the CSP, limited the access of (+)acetonide to the enantioselective binding site. (−)Acetonide has the same access as in the presence of ethanol. The net result is less interaction with the binding site for (+)acetonide compared with (−)acetonide and a consequent reversal of elution order.

The values obtained for $\Delta\Delta H$ and $\Delta\Delta S$, which represent only the enantioselective interactions, lend further credence. The larger absolute values obtained with isopropanol are consistent with one enantiomer having greater access to the enantioselective binding site. Furthermore, for mobile phase B, a reversal of elution order is observed as a function of temperature (Fig. 5). (−)Acetonide is eluted first above 35°C and eluted second below this temperature. At lower temperatures, the tertiary structure of the CSP is more rigid, and the role of steric accessibility would be enhanced.

Effect of pH

The retention and enantioselectivity for ionic compounds are influenced by pH, as electrostatic interactions are believed to play a major role (47–49). The strengths of the electrostatic interactions are influenced by the protolysis of both the solute and the CSP. However, for neutral solutes, changes in pH have been shown to influence enantioselectivity, but with little effect on overall retention (47,49). The effect on enantioselectivity in these cases is attributed to conformational changes in the CSP. The role of pH on enantioselectivity of the CSP for the

Table 1. Effect of Organic Modifier on Thermodynamic Properties of Acetonide

Eluent Composition	ΔH_1 (cal)	ΔH_2 (cal)	$\Delta\Delta H$ (cal)	$\Delta\Delta S$ (calK^{-1})
20% ethanol/0% ipa (A)	−6788	−7069	−281	0.20
9.5% ethanol/6% ipa (B)	−7258	−6152	1106	3.58
0% ethanol/11.5% ipa (C)	−7134	−5070	2064	6.36

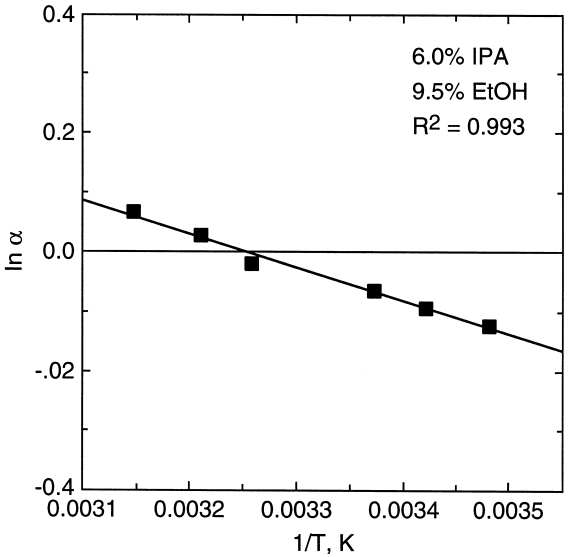

Figure 5. van't Hoff plot for acetonide with 6.0% isopropanol and 9.5% ethanol.

acetonide enantiomers was investigated through variation of pH in the presence of either ethanol or isopropanol.

The pH of the eluent was varied from 2.5 to 7 at a fixed phosphate concentration of 25 m*M*, using ethanol or isopropanol as the modifier. With ethanol as the modifier, both the capacity and selectivity factors increased slightly initially and were relatively constant above pH 4.5. In the presence of isopropanol, a reversal of elution order was observed as a function of pH (Fig. 6). (−)Acetonide is eluted first at low pH, but is eluted second at higher pH, with coelution occurring in between (Fig. 7). Thermodynamic parameters at pH 3 and 7 with isopropanol were generated through van't Hoff plots. At pH 7, $\Delta\Delta H$ and $\Delta\Delta S$ were determined to be 877 cal and 2.48 cal K^{-1}, respectively. At pH 3, $\Delta\Delta H$ and $\Delta\Delta S$ were determined to be −246 calories and −0.41 cal K^{-1}, respectively. The data at high pH are consistent with (−)acetonide having preferential access to the binding site. At lower pH, this preferential access is eliminated, as reflected in the decrease in the overall retention for both enantiomers and the smaller absolute values of the thermodynamic parameters.

At lower pH, the CSP carries less charge as the carboxylic acid groups are protonated. The reduction in charge can lead to conformational changes that more effectively bury the apolar cavity, thus, further reducing access to both enantiomers. It should again be noted that a reduction in capacity factor was seen

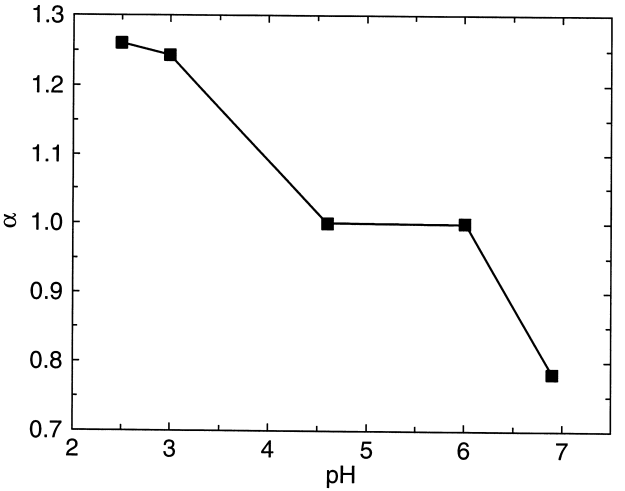

Figure 6. Influence of pH on enantioselectivity with isopropanol as the organic modifier.

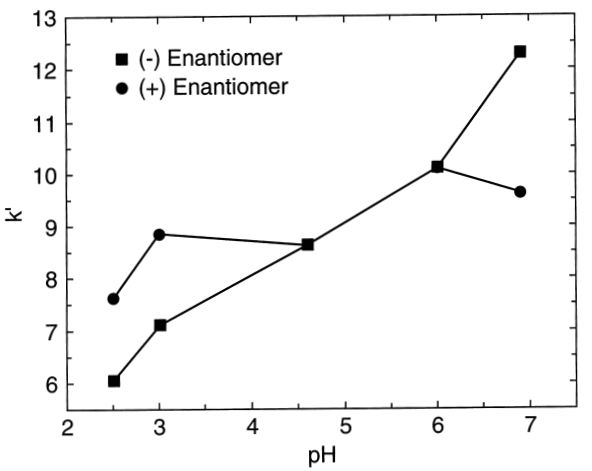

Figure 7. Influence of pH on the capacity factors with isopropanol as the organic modifier.

at low pH for both alcohol modifiers. The enantioselective effect would be more enhanced in the presence of the bulkier alcohol, due to its steric influence.

CONCLUSION

The influence of organic modifier, temperature, and pH on the enantioselectivity of AGP for acetonide has been investigated. These parameters were determined to affect enantioselectivity to the extent that a reversal of elution order could be induced. Our studies found that the role of hydrogen bonding in the chiral discrimination of acetonide was minimal. Rather, an inclusion mechanism was more plausible with the investigated parameters affecting the access to the binding sites, either through induced conformational changes or steric hindrance.

REFERENCES

1. Hermansson, J. Trends Anal. Chem. **1989**, *8*, 251.
2. Allenmark, S. J. Liq. Chromatogr. **1986**, *9*, 425.
3. Miwa, T.; Miyakawa, T.; Kayano, M.; Miyake, Y. J. Chromatogr. **1987**, *408*, 316.
4. Herskovits, T.; Gadegbeku, B.; Jaillet, H. J. Biol. Chem. **1970**, *245*, 2588.
5. Jirgensons, B. Biochim. Biophys. Acta **1973**, *328*, 314.
6. Jirgensons, B. Biochim. Biophys. Acta **1976**, *434*, 58.
7. Enquist, M.; Hermansson, I. J. Chromatogr. **1990**, *519*, 271.
8. Hermansson, J.; Hermansson, I. J. Chromatogr. A **1994**, *666*, 181.
9. Aubert, J.; Biserte, G.; Loucheux-Lefebvre, M. Arch. Biochem. Biophys. **1976**, *175*, 410.
10. Borga, O.; Piafsky, K.; Nilsen, O. Clin. Pharmacol. Ther. **1977**, *22*, 539.
11. Piafsky, K. Clin. Pharmacokinet. **1980**, *5*, 246.
12. Maruyama, T.; Otagiri, M.; Takadate, A. Chem. Pharm. Bull. **1990**, *6*, 1688.
13. Imamura, H.; Maruyama, T.; Otagiri, M. Biol. Pharm. Bull. **1993**, *9*, 926.
14. Muller, W.; Stillbauer, A. Arch. Pharmacol. **1983**, *322*, 170.
15. Otagiri, M.; Yamamichi, R.; Maruyama, T.; Imai, T.; Suenaga, A.; Imamura, Y.; Kimachi, K. Pharm. Res. **1989**, *2*, 156.
16. Aki, H.; Yamamoto, M. J. Pharm. Pharmacol. **1989**, *41*, 674.
17. Urien, S.; Bree, R.; Tests, B.; Tilliment, J. Biochem. J. **1993**, *289*, 767.
18. Rojo-Domingues, A.; Hernandez-Arana, A. Protein Seq. Anal. Data **1993**, *5*, 349.
19. Aubert, J.; Loucheux-Lefebvre, M. Arch. Biochem. Biophys. **1976**, *175*, 400.

20. Fehske, K.; Muller, W.; Wollert, U. Mol. Pharmacol. **1979**, *16*, 778.
21. Fehske, K.; Muller, W.; Wollert, U. Biochem. Pharmacol. **1981**, *30*, 687.
22. He, X.; Carter, D. Nature (London) **1992**, *358*, 209.
23. Chattopadhyay, A.; Tian, T.; Kortum, L.; Hage, D. J. Chromatogr. B **1998**, *715*, 183.
24. Brunner, F.; Muller,W. J. Pharm. Pharmacol. **1987**, *39*, 986.
25. Oravcova, J.; Bystricky, S.; Trnovec, T. Biochem. Pharmacol. **1989**, *16*, 2575.
26. Campbell, D. Eur. J. Drug Metab. Pharmacokinet. **1990**, *15*, 109.
27. Fitos, I.; Visy, J.; Simonyi, M. Biochem. Pharmacol. **1991**, *3*, 377.
28. Halsal, H.; Kirley, T. Biochem. Biophys. Res. Commun. **1981**, *2*, 761.
29. El-Gamal, S.; Wollert, U.; Muller, W. J. Pharm. Pharmacol. **1982**, *34*, 152.
30. Busby, T.; Ingham, K. Biochim. Biophys. Acta **1986**, *871*, 61.
31. Rojo-Dominguez, A.; Zubillaga-Luna, R.; Hernandez-Arana, A. Biochemistry **1990**, *29*, 8689.
32. See-Waters, M.; Sidler, D.; Simon, A.; Middaugh, C.; Thompson, R.; August, L.; Bicker, G.; Perpall, H.; Grinberg, N. Chirality **1999**, *11*, 224.
33. Narayanan, S. J. Pharm. Biomed. Anal. **1992**, *10*, 251.
34. Karlsson, A.; Pettersson, K.; Hernqvist, K. Chirality **1995**, *7*, 147.
35. Hermansson, J.; Grahn, A. J. Chromatogr. A **1995**, *694*, 57.
36. Hermansson, J. J. Chromatogr. **1983**, *269*, 71.
37. Fitos, I.; Visy, J.; Simonyi, M.; Hermansson, J. J. Chromatogr. A **1995**, *709*, 265.
38. Schill, G.; Wainer, I.; Barkan, S. J. Liq. Chromatogr. **1986**, *9*, 641.
39. Haupt, D.; Pettersson, C.; Westerlund, D. Fresenius J. Anal. Chem. **1995**, *352*, 705.
40. Li, F.; Cooper, S.; Cote, M.; Ayotte, C. J. Chromatogr. B **1994**, *660*, 327.
41. Ho, M.; Erhard, K.; Chen, T. J. Liq. Chromatogr. **1994**, *17*, 761.
42. Enquist, M.; Hermansson, J. J. Chromatogr. **1990**, *519*, 285.
43. Williams, R.; Edwards, J.; Potter, M. J. Liq. Chromatogr. **1993**, *16*, 171.
44. Fitos, I.; Visy, J.; Simonyi, M.; Hermansson, J. J. Chromatogr. **1992**, *609*, 163.
45. Hermansson, J.; Hermansson, I. J. Chromatogr. A **1994**, *666*, 181.
46. Zepeda, A.; Barman, B.; Martire, D. Anal. Chem. **1992**, *64*, 1978.
47. Hermansson, J. J. Chromatogr. **1983**, *269*, 71.
48. Hermansson, J.; Ericksson, M. J. Liq. Chromatogr. **1986**, *9*, 621.
49. Wainer, I. Jadaud, P.; Schombaum, G. Chromatographia **1988**, *25*, 903.

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