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Journal of Pharmaceutical and Biomedical Analysis 46 (2008) 898–906
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
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Comparative study of immobilized α1 acid glycoprotein and ovomucoid protein stationary phases for the enantiomeric separation of pharmaceutical compounds

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Received 8 February 2007; received in revised form 5 July 2007; accepted 8 July 2007
Available online 12 July 2007

Abstract

The enantioselectivity of two protein chiral stationary phases, α1 acid glycoprotein (AGP) and ovomucoid protein (OVM) are compared. Neutral, basic and acidic pharmaceutical compounds were screened on both stationary phases. Selected parameters such as mobile phase pH, temperature, and organic modifier were varied in order to achieve chiral separations. Relations between the enantioselectivities of the two stationary phases and the properties of the compounds (acidity, basicity, structure of molecule) were also investigated. The OVM column tends to separate larger molecules better than the AGP column. Reversal of elution order for some compounds was observed on the two columns under similar experimental conditions, or with the same column as a function of pH and organic modifier. Many practical aspects were also discussed.

Keywords: α1 acid glycoprotein (AGP); Ovomucoid protein (OVM); Pharmaceutical compounds; LC-stationary phases; Reversed phase liquid chromatography (RPLC)

1. Introduction

Chiral analysis of pharmaceutical compounds is very important for the pharmaceutical industry since enantiomers of drug compounds may possess quite different pharmacological and toxicological properties. In the past two decades, high performance liquid chromatography (HPLC) with reversed phase (RP) and normal phase (NP) modes have been the most popular techniques used for enantiomeric separations. The applications of these techniques have been extensively covered in recent publications [1–5]. Although NPLC has been widely used, interest in the chiral separation in RPLC has always been high in pharmaceutical API process industries due to its advantages. For example, RPLC provides good solubility for polar compounds, which are often included in API synthetic reaction mixtures. More importantly, RPLC mobile phase provides a low UV cut off and thus high detection sensitivity, which can be used to control enantiomeric impurities to levels as low as 0.1% based on ICH guidelines.

Numerous chiral stationary phases (CSPs) have been developed for RPLC. These CSPs include Pirkle, protein, crown ether, ligand-exchange, macrocyclic antibiotics, cyclodextrins and polysaccharide derivatives. Protein CSPs are the most mature relative to all of these CSPs for RPLC [1,5]. Noteworthy amongst these protein CSPs is α1 acid glycoprotein (AGP), first introduced by Hermansson in 1983 [6]. Several protein based CSPs have been commercially available, including AGP, ovomucoid protein (OVM) [7], bovine serum albumin (BSA) [8], human serum albumin (HSA) [9], and cellobiohydrolase I (CBH I) [10]. Proteins play a major physiological role with their ability to reversibly bind small drug molecules. Because they possess a large amount of functional groups, proteins are able to bind with a wide range of substances through hydrophobic or electrostatic interactions. The inherent chirality of proteins allows for enantioselective interactions with chiral compounds. The protein-based CSPs have advantages such as a wide range of enantioselectivity, applicability, and compatibility with the aqueous buffered mobile phase of RPLC. Therefore, such CSPs have been widely used in the pharmaceutical industry [1,5–10].

In the past two decades, our laboratory has extensively utilized AGP and OVM CSPs for a wide range of pharmaceutical

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doi:10.1016/j.jpba.2007.07.008

compounds from basic to acidic to neutral. AGP and OVM have distinguishing features. The major differences are their molecular weights and isoelectric points (p*I*). These differences affect their separation behaviors due to the important role that the charge status of the protein plays for enantio-recognitions (which are directly related to the protein's isoelectric points). It was believed that the protein domain of the molecule could form a hydrophobic pocket or cleft [11]. The size of the pocket might be different for AGP versus OVM due to differences in their molecular weights [12] resulting in different enantioselectivity.

In this paper, a general chiral RPLC method development strategy utilizing AGP and OVM CSPs together with a successful method for enantiomeric separation of numerous drug related compounds is presented. Many practical aspects are discussed through specific examples in order to demonstrate how to develop and validate a sensitive and rugged separation method. Relations between the enantioselectivity of the two CSPs and the nature of the compounds were demonstrated and the separation mechanism was briefly attributed.

2. Experimental

2.1. Reagents

All organic solvents used in the study were HPLC grade and purchased from Fisher Scientific (Fairlawn, NJ, USA). Phosphoric acid (85%) was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). Water used in the study was deionized water purified through a Millipore deionization device (Milford, MA, U.S.A.). Chemicals including compound-a to compound-z (Fig. 1) were prepared and well characterized by the Process

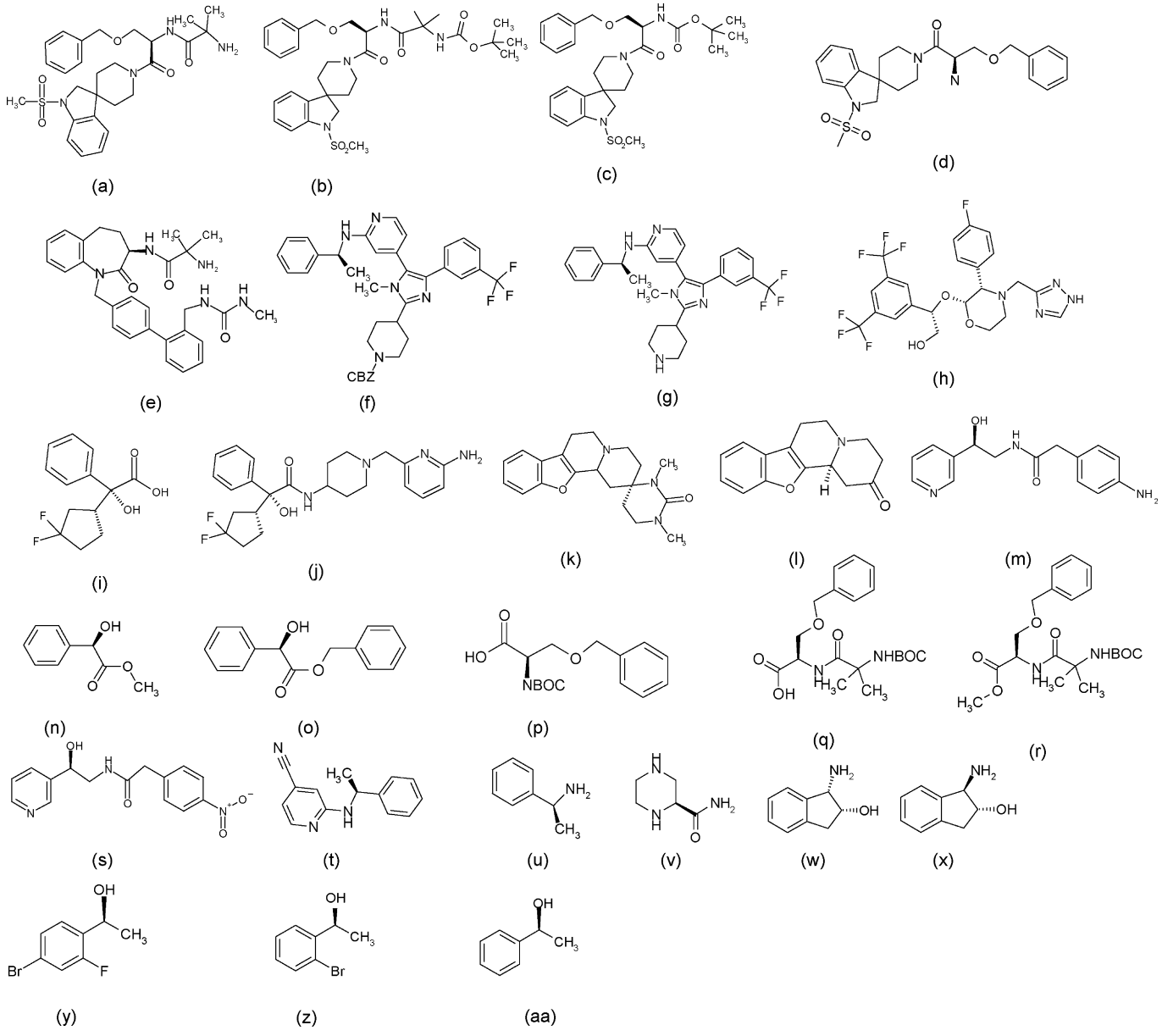

Fig. 1. Structures of studied compounds.

Research Department of Merck Research Laboratories (Rahway, NJ, USA). Compound-aa (Fig. 1) was purchased from Aldrich (Milwaukee, WI, USA).

2.2. Instrumentation

An Agilent 1100 system with diode array UV detection and thermal-controller was used. All chromatograms were processed by a Turbchrom data system equipped with Access*Chrom software (Version 1.9) (PE Nelson. Cupertino. CA, USA).

2.3. Chromatographic columns

The columns were Chiral-AGP (15 cm in length and 4.0 mm i.d.) and ULTRON ES-OVM (15 cm in length and 4.6 mm i.d.). Chiral-AGP column was purchased from Regis Chemical Co. (Morton Grove, IL, USA) and ULTRON ES-OVM was purchased from Agilent Technologies Co. (Wilmington, DE, USA).

2.4. Chromatographic conditions

All LC separations, except where specified, were performed at a temperature of 25 °C. The mobile phases were gradient for the screening and then isocratically pump-mixed at specified compositions for optimized separation. The flow-rate was varied from 0.8 to 1.0 mL/min; the injection volume was 10 μL; the detection was UV at 205 nm in all cases. The retention factor k for the two bands (formerly referred to as the capacity factor k') was determined as $k = (t_R - t_0)/t_0$, where t_R and t_0 were retention times of retained and un-retained compounds, respectively. In this study, t_0 was estimated based on the solvent peak.

2.5. Preparation of sample solutions

The samples were dissolved in a diluent mixed with acetonitrile and water at a 1:1 ratio. The concentrations of samples were varied from 0.1 to 0.5 mg/mL.

3. Results and discussion

3.1. Selection of studied compounds

Based on the literature, protein columns can be used for separations of an extremely broad range of enantiomers including amines, acids, and neutral compounds. Therefore, a wide range of drug-related chiral compounds with diversified structures (as shown in Fig. 1) was selected for the studies. All compounds were screened on both AGP and OVM chiral stationary phases. Then, the enantioseparation for each compound was optimized by varying mobile phase pH, organic modifiers, column temperature, and buffer ionic strength.

3.2. Parameters affecting chiral resolutions

3.2.1. Effect of pH

One of most important parameters for enantiomeric separation on protein columns is the pH of the mobile phase, since the effective charges on both ionizable enantiomers and protein stationary phases are greatly affected by pH. AGP has an isoelectric point (p*I*) of 2.7 and OVM has a p*I* of 4.1 [1,5]. Both columns are expected to have a net negative charge on the stationary phases when the mobile phase pH is above the p*I* values. Reducing the mobile phase pH towards the p*I* points reduces the negative charges of the stationary phases, resulting in shorter retention for the basic compound. In contrast, increasing the mobile phase pH towards the p*I* reduces the positive charge of the stationary phases, resulting in shorter retention for the acidic compounds. In this study, the roles of pH on enantioselectivity of the protein CSP for different compounds were investigated by varying mobile phase pH in the presence of organic modifier.

Fig. 2a and b illustrates the effects of the pH on the retention factors of compound-l. The pH of mobile phase (A) was varied over a range from 3.5 to 7.5. Acetonitrile was used as an organic modifier at a fixed ratio of 15%. Retention factors k of enantiomers and enantioselectivity (α) increased on both AGP and OVM columns as the pH of the mobile phase increased, since compound-l is a base. As Fig. 2a and b shows, at a pH of 3.5, no separation occurred on the OVM column since the

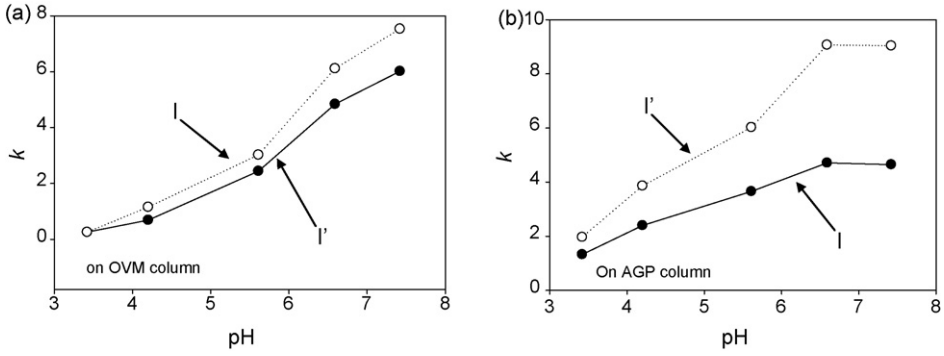

Fig. 2. (a) Effect of mobile phase pH for basic compound-l on the OVM column; (b) effect of mobile phase pH for basic compounds-l on the AGP column. Mobile phase conditions: MeCN/phosphate buffer (15 mM, pH values are varied), ratio: 15/85 (v/v). Flow rate: 1.0 mL/min. UV wavelength: 205 nm. Column temperature: 25 °C. Injection volume: 10 μL.

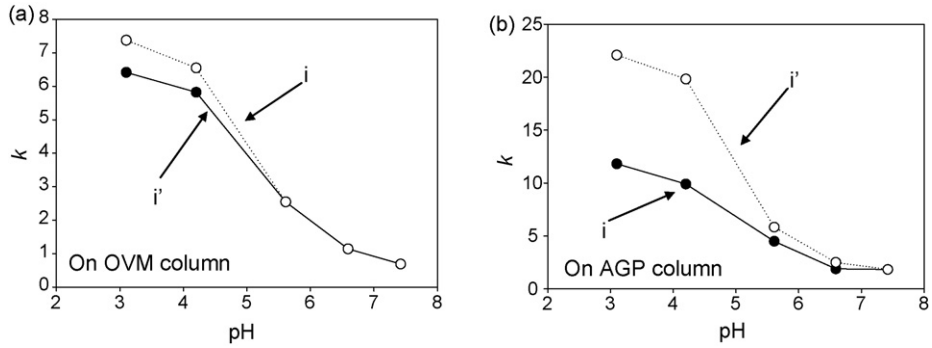

Fig. 3. (a) Effect of mobile phase pH for acidic compound-i on the OVM column; (b) effect of mobile phase pH for acidic compound-i on the AGP column. Mobile phase conditions: MeCN/phosphate buffer (15 mM, pH values are varied), ratio: 15/85 (v/v). Flow rate: 1.0 mL/min. UV wavelength: 205 nm. Column temperature: 25 °C. Injection volume: 10 μL.

pH value of the mobile phase was below the PI value of the stationary phase. However, separation did occur on the AGP column since the pH value of the mobile phase is above the PI value of the stationary phase. The contrasting behaviors were observed for acidic compound-i. As can be seen from Fig. 3a and b, both the retention factors k of enantiomers and the enantioselectivity (α) decreased on both AGP and OVM columns as the pH of the mobile phase increased, since compound-i is an acid. Compound-i is present in a partially anionic form when the pH value of the mobile phase is above its pK_a of 4.3. Unfortunately, both OVM and AGP stationary phases are also negatively charged in that pH range. Thus, no ionic binding could be formed due to the repulsions. However, the degree of net negative charge of both analyte and stationary phases would be reduced as mobile pH decreased, resulting in retention due to the reduction of the repulsion between the analyte and the protein CSP. The enantioseparation seems to have occurred through hydrophobic interaction and hydrogen bonding.

It is interesting that reversal in the elution orders of both compound-l and compound-i was observed from AGP column to OVM column despite the fact that both were under similar mobile phase conditions.

Although the recommended pH range of the mobile phase for the protein columns is 3–7.5, this study explored a higher pH mobile phase with a borate buffer, since it is known that many silica-based columns can be maintained with such a buffer. Experiments were performed by varying the pH of the mobile phase (A) over a range from 6.0 to 9.3 with the presence of acetonitrile on both AGP and OVM columns. A reversal of elution order was observed as a function of pH for compound-j on the AGP column as shown in Fig. 4. Enantiomer-j is eluted first at pH of 6.5, but is then eluted second at pH of 9.3. The isoelution point was observed at pH of 8.3. More importantly, the column is stable at pH of 9.3 with a borate buffer. More than 100 injections of racemate of compound-j were made on two AGP columns over a 2-week period. The variation on retention times was ±0.2 min. After being used in stability studies, both columns were still able to separate other enantiomers at lower pH level. The data suggested that a possible reversible conformational change of immobilized AGP occurred between pH 6.5 and 9.3. A similar exploration on the OVM column was not successful due to the poor stability of the column at pH 9.3. Such differences between the AGP and OVM columns may be due to the fact that the AGP column is cross-linked.

3.2.2. *Effect of temperature*

In order to further understand the mechanism involved in the binding of enantiomers to both AGP and OVM columns, temperature studies of compound-l, compound-h, and compound-j were performed under various mobile phase pH levels. The thermodynamic parameters involved under each mobile phase condition can be determined by using the known van't Hoff equation [13] as described below,

$$\ln(k) = -\left(\frac{\Delta H^\circ}{RT}\right) + -\left(\frac{\Delta S^\circ}{R}\right) + \ln \Phi$$

where k is the retention factor, R the ideal gas constant, T the absolute temperature, ΔH° the difference in enthalpy of the solute in the two phases, ΔS the difference in entropy of the solute in the two phases, and Φ is the solute phase ratio. A plot of ln(k) versus the reciprocal of the absolute temperature is linear with the slope representing the enthalpic term and the intercept representing the entropic term. If a pair of enantiomers with retention factor k_1 and k_2 are considered in an enantiomeric separation, the van't Hoff equation can be derived for a similar

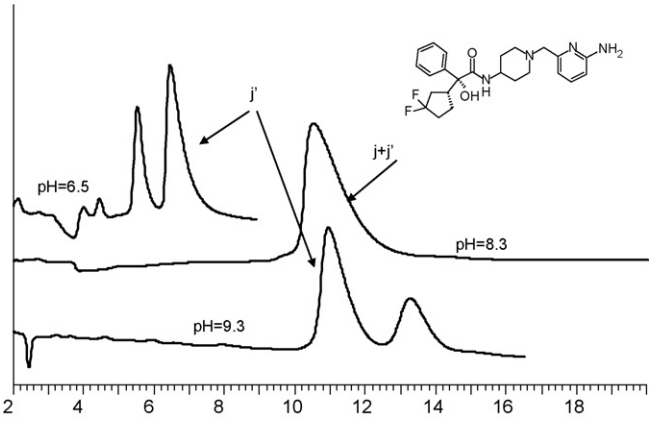

Fig. 4. Effect of mobile phase pH on the elution order for compound-j. Mobile phase conditions: MeCN/phosphate buffer (15 mM, pH values are varied), ratio: 10/90 (v/v). Flow rate: 1.0 mL/min. UV wavelength: 205 nm. Column temperature: 25 °C. Injection volume: 10 μL.

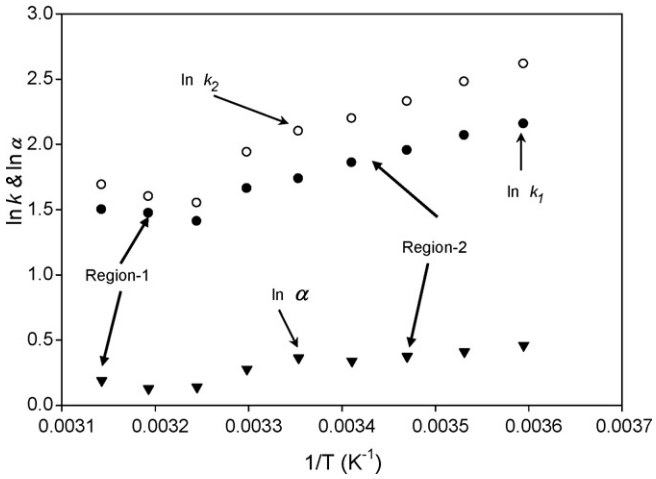

Fig. 5. van't Hoff plots for compound-l on the OVM column at mobile phase pH 6.5.

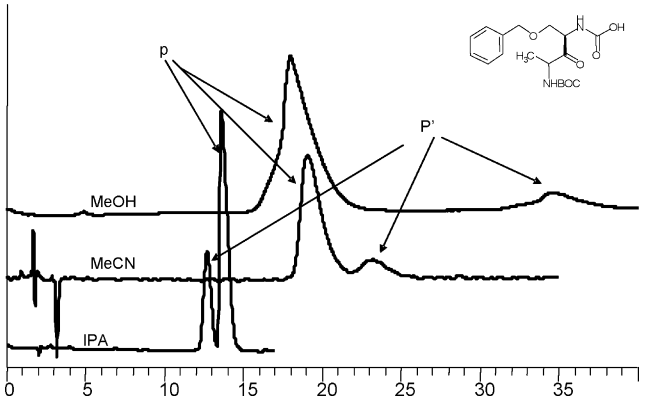

Fig. 6. Effect of mobile phase pH on the elution order for compound-p. Column: AGP; mobile phase conditions: mobile phase A: pH 2.5 phosphate buffer (15 mM), mobile phase B: MeCN and IPA; ratio: 10/90 (v/v). Flow rate: 1.0 mL/min. UV wavelength: 205 nm. Column temperature: 25 °C. Injection volume: 10 μL. Solution was prepared by mixing of two pure enantiomers in different ratio.

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

$$\ln\left(\frac{k_2}{k_1}\right) = \ln(\alpha) = -\left(\frac{\Delta\Delta H^\circ}{RT}\right) + -\left(\frac{\Delta\Delta S^\circ}{R}\right)$$

Thermodynamic parameters at different pH levels of mobile phases were obtained from van't Hoff plots. The temperature range was from 5 to 45 °C at 5° intervals. All plots generated under low pH mobile phases for both ln(k) and ln(α) on both OVM and AGP stationary phases were linear. This linearity indicated that, under the designed mobile phase conditions, there is no change over the temperature range of the interactions of the enantiomers with the stationary phases. However, the van't Hoff plots of both ln(k) and ln(α) for both compound-l and compound-h on the OVM column with pH 6.5 mobile phase are non-linear with a transition around 35 °C. Examples of plots for compound-l on the OVM column with pH 6.5 mobile phase are shown in Fig. 5.

Thermodynamic parameters calculated based on the van't Hoff plots are listed in Table 1. Most of the $\Delta\Delta H^\circ$ and $\Delta\Delta S^\circ$ values are negative, the data suggests that enantio-separation under low pH mobile phases is an enthalpy-driven process, regardless of organic modifier type for either AGP or OVM stationary phases. Positive values were observed for compound-l on the OVM column with pH 6.5 mobile phase at the high temperature region, regardless of organic modifier type. This behavior is more or less specific to the OVM column, since similar observations were obtained for compound-h. Positive values were also obtained for the AGP column with pH 9.3 mobile phase. All of this data suggests the occurrence of entropy-driven separations and may reflect a reversible conformational change in the immobilized proteins [1,5].

3.2.3. *Effect of organic modifiers*

Four common organic modifiers including acetonitrile (MeCN), methanol (MeOH), ethanol (EtOH), and isopropanol (IPA) were screened for all selected compounds on both OVM and AGP columns. It was found that peak shape is sharper with less retention time when MeCN is used. The retentions of the solutes increase in the order of EtOH > MeOH > MeCN in most cases. Such behavior is usually observed on achiral columns and is attributed to increasing viscosity. However, the behaviors of IPA in several cases cannot be explained by viscosity. As can be seen from Fig. 6, the enantiomer-p is eluted first with MeCN and then with IPA. Elution order is the same with both MeCN and EtOH and retention times of both enantiomers are much shorter with IPA. Similar behaviors were observed for compound-i as well. Reversal of elution order by switching from MeCN to IPA has been reported in the literature for warfarin with AGP [14]. A similar reversal of elution order for an alkaloid has also been reported using the HAS-based column [15]. The reversal of the affinity patterns of enantiomers towards chiral selectors depending on organic modifier types has been attributed to several reasons. The first reason is that the modifier is competing with solute enantiomers when binding to AGP. With MeCN as the modifier, the solute can interact with the protein through stronger hydrogen binding. With IPA as the modifier, the solute's ability to interact through hydrogen bonding with the protein is reduced due to competition with IPA. Therefore, other types of interactions such as dipole–dipole interaction may have different affinities for the enantiomers, resulting in a reversal of elution order. The second reason is that the modifiers induce reversible changes in the tertiary structure of the protein in such a way as to reverse the enantioselectivity.

Our experimental results do not support the first reason, since modifiers with similar hydrogen bonding properties to IPA, such as MeOH and EtOH, nevertheless did not show similar behaviors to IPA. We do not yet have a complete understanding of the elution order reversal phenomenon, but it appears that it may be due to the bulkiness of IPA. Such bulkiness may restrict certain bonding sites of the AGP and hence further induce change in the elution order. It is important to emphasize that such restriction is only for specific bonding sites, because a small change in structure may eliminate such effects. For example, in Fig. 1, compound-i and compound-j contain similar chiral centers; the only difference is that one of the side-chains on the chiral cen-

Table 1
Influence of different parameters on $\Delta\Delta H^{\circ}$ and $\Delta\Delta S^{\circ}$

Column/pH/organic modifier/compound/region	$\Delta\Delta H^{\circ}$ (kcal/mol)	$\Delta\Delta S^{\circ}$ (cal mol^{-1} K (kcal/mol))
AGP/4.0/MeCN/compound-l	−2.51	−0.4
AGP/4.0/MeOH/compound-l	−1.52	−0.1
AGP/4.0/EtOH/compound-l	−2.21	−0.5
AGP/4.0/IPA/compound-l	−0.31	−0.1
AGP/7.0/MeCN/compound-l	−0.63	−0.1
AGP/7.0/MeOH/compound-l	−0.31	−0.2
AGP/7.0/EtOH/compound-l	−2.01	−0.3
AGP/7.0/IPA/compound-l	−0.65	−0.4
AGP/6.5/compound-j	−0.21	−0.1
AGP/9.3/compound-j	+2.51	+1.2
OVM/4.0/MeCN/compound-l	−4.11	−3.2
OVM/4.0/MeOH/compound-l	−2.60	−0.3
OVM/4.0/EtOH/compound-l	−3.51	−1.2
OVM/4.0/IPA/compound-l	−2.02	−0.3
OVM/7.0/MeCN/compound-l/region-1	+2.91	+0.1
OVM/7.0/MeCN/compound-l/region-2	−3.11	−0.1
OVM/7.0/MeOH/compound-l/region-1	+0.61	+1.2
OVM/7.0/MeOH/compound-l/region-2	−1.20	−0.5
OVM/7.0/EtOH/compound-l/region-1	+0.31	+0.6
OVM/7.0/EtOH/compound-l/region-2	−3.01	−1.5
OVM/7.0/IPA/compound-l/region-1	+0.11	+0.4
OVM/7.0/IPA/compound-l/region-2	−1.12	−0.4
OVM/4.0/MeCN/compound-h	−2.21	−0.2
OVM/7.0/MeCN/compound-h/region-1	+0.32	+1.5
OVM/7.0/MeCN/compound-h/region-2	−3.51	−3.4

ter is carboxylic acid for compound-i but is replaced by an aminopyridin-piperidin functional group for compound-j. Previously, we reported that similar enantioselectivity can be obtained for compound-j using an AGP column with a variety of organic modifiers including MeCN, MeOH, EtOH and IPA [16]. However, in this study, we found that elution orders were reversed from MeCN to IPA for compound-i although compound-i had a similar chirality to compound-j. Similar reversal elution order behavior was not observed on the OVM column under the same experimental conditions, thus supporting the hypothesis that such organic modifier effects occur on specific AGP protein sites.

The effect of each modifier concentration in the range from 3% to 25% on the enantioseparation of compound-l was also investigated at a mobile phase pH of 6.5 on both AGP and OVM columns. The retention factors for both enantiomers of compound-l decreased as the ratio of each modifier increased. Plots of $\log k$ versus % modifier (MeCN, MeOH, EtOH and IPA) were all linear. Such linear trends can be attributed to the decrease of hydrophobic interactions between each enantiomer and protein stationary phase as the mobile phases become less polar. These behaviors indicated that the hydrophobic interactions played a major role in the enantiomeric separation of compound-l.

3.2.4. Effect of phosphate buffer concentration

The effect of buffer concentrations was studied on the AGP column using enantiomers of compound-m. The concentration of the phosphate buffer was varied from 5 to 50 mM at pH of 6.5 while the MeCN ratio was kept at 10%. The retention factors of both enantiomers of compound-m were increased as the concentration of the buffer increased with improvements in resolution.

Temperature studies of compound-m in the range of 5–45 °C were performed with 5, 25, and 50 mM concentrations of phosphate buffers. It was noticed that van't Hoff plots were linear at 5 and 25 mM but were non-linear at 50 mM with a transition around 35 °C. Thermodynamic values at each buffer concentration were obtained from van't Hoff plots. At a low buffer concentration of 5 mM, both $\Delta\Delta H^{\circ}$ and $\Delta\Delta S^{\circ}$ values are more negative. But these values become less negative as buffer concentrations increase. Positive values of $\Delta\Delta H^{\circ}$ and $\Delta\Delta S^{\circ}$ were obtained at high temperature regions when the buffer concentration reached to 50 mM. These observations clearly indicated that electrostatic interactions also played a role in the chiral discriminations on the AGP column.

3.3. Effect of the compound structure

Table 2 is a summary of data obtained under optimum chromatographic conditions for the 27 pharmaceutical compounds with the AGP and OVM columns. The retention time and selectivity of each pair of enantiomers are listed. The results clearly indicated that enantioseparations on both AGP and OVM stationary phases are structurally dependent. Comparisons of these results reveal some interesting trends.

3.3.1. Effect of molecular size

The overall trends of Table 2 clearly demonstrate that enantioseparation is affected by molecular size. No enantioselectivity was observed for small amine molecules on both AGP and

Table 2
Optimum enantioseparation of studied compounds on AGP and OVM columns

Compound ID	AGP			OVM		
	Selectivity	t (min)	t' (min)	Selectivity	t (min)	t' (min)
a_{MP1}	1.00	28.86	28.86	1.09	25.07	27.09
b_{MP1}	1.00	32.14	32.14	1.39	9.43	12.51
c_{MP1}	1.00	24.22	24.22	1.56	9.82	14.51
d_{MP1}	1.00	20.12	20.12	1.17	7.01	7.95
e_{MP1}	1.00	30.21	30.21	1.56	12.21	13.02
f_{MP1}	1.00	12.32	12.32	1.63	8.96	25.15
g_{MP1}	1.00	21.15	21.15	1.34	7.52	9.76
h_{MP1}	1.00	19.56	19.56	1.35	3.32	3.95
$*i_{MP3}$	1.85	19.34	34.56	1.25	12.76	10.93
$*j_{MP1}$	1.20	8.34	10.02	1.20	8.68	7.23
j_{MP5}	1.24	11.2	13.5	–	–	–
$*k_{MP1}$	1.39	8.50	11.21	1.83	9.82	6.07
$*l_{MP1}$	1.36	7.02	9.01	1.53	7.25	5.25
m_{MP1}	1.34	4.75	5.96	1.28	7.12	8.67
n_{MP3}	1.46	6.20	8.34	1.00	6.35	6.35
o_{MP4}	1.72	6.34	9.83	1.00	7.23	7.23
p_{MP4}	1.65	3.56	8.25	1.00	6.56	6.56
q_{MP1}	1.52	5.67	7.85	1.00	6.21	6.21
r_{MP1}	1.26	7.89	9.56	1.00	8.34	8.34
s_{MP1}	2.06	3.21	5.02	1.00	6.25	6.25
t_{MP1}	1.24	9.45	11.34	1.00	9.56	9.56
u_{MP1}	1.00	15.62	18.72	1.00	12.34	12.34
v_{MP1}	1.00	6.34	6.34	1.00	4.21	4.21
w_{MP1}	1.00	4.56	4.56	1.00	4.02	4.02
x_{MP1}	1.00	3.12	3.12	1.00	3.01	3.01
y_{MP1}	1.00	5.23	5.23	1.66	4.56	6.58
z_{MP1}	1.00	4.12	4.12	1.27	3.65	4.23
aa_{MP1}	1.00	3.88	3.88	1.00	3.15	3.15

Mobile phase (MP)1: MeCN/phosphate buffer (15 mM, pH 6.5), ratio: varied. MP2: IPA/phosphate buffer (15 mM, pH 6.5), ratio: varied. MP3: MeCN/phosphate buffer (15 mM, pH 3.1), ratio: varied. MP4: MeCN/phosphate buffer (15 mM, pH 4.1), ratio: varied. MP5: MeCN/borate buffer (15 mM, pH 9.3), ratio: varied. Compounds showing a selectivity of 1.00 in table gave a selectivity of 1.00 at all of the above conditions.
*The elution orders are reversed on two columns.

OVM columns (compound-u to compound-x and compound-aa). Greater enantioselectivity was generally observed for larger molecules with the OVM column (compound-a to compound-h, compound-y to compound-z) and for smaller molecules with the AGP column (compound-n to compound-t). Enantiomeric separations were achieved for molecules with intermediate sizes on both AGP and OVM columns (compound-i to compound-m). It is worth noting that 4 out of 5 enantioseparations showed reversal elution orders from AGP to OVM. These results clearly demonstrated that AGP and OVM columns provide complimentary enantioselectivities.

3.3.2. Effect of non-enantioselective interactions

In general, the longer retentions indicated a stronger overall bonding between the solutes and stationary phases. However, stronger overall bonding may not lead a better enantioresolution if the bonding is not enantioselective. For example, compound-a, compound-b, and compound-c (shown in Fig. 1) are structurally closely related. All three compounds are relatively large and were only separated on the OVM column. The overall mass of compound-b is higher than compound-a since it contains a BOC protection group. However, the retention of compound-b is less than the retention of compound-a with a better enantioselectivity. This observation can be attributed as an effect of non-enantioselective interactions. The amino group of compound-a is far away from the chiral center, but it interacts with the OVM stationary phase strongly through an electrostatic interaction. When the amino group is protected, such non-enantioselective interaction is reduced, thus enantioselectivity is enhanced while overall retention is reduced. Compared with compound-b, the side chain of compound-c is further reduced, therefore the enantioselective interaction between the CSP and nitrogen (oxygen) around the chiral center is further enhanced, resulting in a better enantioselectivity.

3.3.3. Effect of charge status

As discussed in the above sections, proteins are amphibious in terms of charge status. Both AGP and OVM are considered acidic proteins [17]. Therefore, it is recommended that basic enantiomers be separated at mobile phase pH > pI and that acidic enantiomers be separated at mobile phase pH < pI to enhance electrostatic interactions. This is only true when the ionic sites are located in the vicinity of the chiral center. In the case where ionic sites are located far away from the chiral center, stronger overall retention may yield poorer enantioselectivity due to non-specific interaction. The difference between the enantioselectivity of compound-i and the enantioselectivity of compound-j is a good example. As discussed in Section

3.2.2, both compound-i and compound-j contain similar chiral centers. Compound-i possesses an acidic site in the vicinity of its chiral center while compound-j contains a primary amine far away from its chiral center. With pH 3 mobile phase for compound-i and pH 6.5 mobile phase for compound-j, the overall electrostatic interactions for both compounds are enhanced. However, the enantioseparation of compound-i is much better than that of compound-j since the electrostatic interactions between the primary amine of compound-i and its protein sites are not enantio-specific. It is interesting that elution orders of compound-i and compound-j were reversed on the same columns, clearly indicating that chiral recognitions occurred on different sites of the proteins.

3.3.4. Effect of hydrophobicity

Since enantiomeric separation using AGP and OVM columns are done under reversed phase HPLC (RP-HPLC) mode, hydrophobic interactions are one of the major driving forces for enantioselective discrimination. The data listed in Table 2 for compound-y, compound-z, and compound-aa confirmed the importance of such hydrophobic interactions. All three compounds are structurally closely related. Each compound differs only by the halogen substituent groups on the aromatic ring. Compound-y contains both fluoro and bromide subsituents, compound-z has only a bromide subsituent and compound-aa has no halogen substituent. It is known that retention under RP-HPLC mode can be increased by adding halogen substituent groups on the aromatic ring, due to the increasing hydrophobicity of the molecule [18]. As Table 2 shows, the addition of fluoro and bromide substituents on the aromatic ring resulted in increased retention times and enantioselectivity for compound-y and compound-z. The degree of increase is in alignment with the increase of hydrophobicity.

3.4. Method validation aspects

It is important that developed chiral methods can be validated. Some useful practical aspects are presented in the following sections.

3.4.1. Elution order

One of the most important and challenging aspects for LC-chiral method validation is the limit of detection (LOD). In general, the peaks obtained using protein columns are relatively broad. Therefore, it is vital to have the minor enantiomer elute in the front of the major enantiomer so as to avoid interference from the tailing of the major enantiomer in order to enhance the LOD. As discussed above, the elution orders of enantiomers can be reversed through different ways, such as varying organic modifiers, mobile phase pHs, and columns. Therefore, it is recommended that, during the initial method development, an automated screen system be set up to control all variables, such as establishing a mobile phase pH at 2.5 and 6.5 with both MeCN and IPA and both AGP and OVM columns. Then, an optimized method with a desired elution order can be selected to achieve an adequate LOD. Fig. 7 is an example, showing a 0.1% LOD chiral separation method for compound-i.

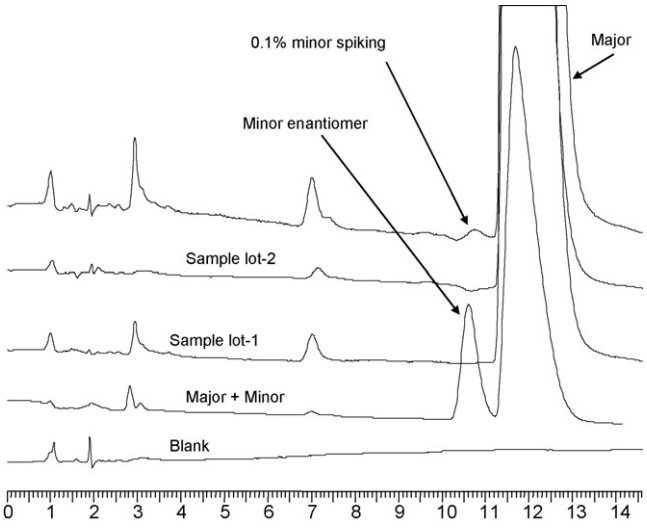

Fig. 7. Chromatograms obtained under optimized LC conditions for compound-i. HPLC conditions: column: OVM, 150 mm × 4.6 mm, 5 μm; column temperature: 25 °C; mobile phase A: 15 mM pH 2.5 phosphate buffer; mobile phase B: MeCN; isocratic run with ratio at A/B = 90/10; flow rate: 1.0 mL/min, UV wavelength: 205 nm. Column temperature: 25 °C. Injection volume: 10 μL.

3.4.2. Selection of buffers for the mobile phase

It is worth noting that the UV cut off should be carefully considered in order to obtain adequate LOD since peak efficiencies are in general relatively low for protein columns. Enantioresolution often suffers as a result of loading higher sample sizes. Therefore, a mobile phase with low background UV absorbance is preferred to enhance the LOD. We have previously reported a comparison signal to noise (S/N) ratios at wavelengths of 200 and 220 nm using different buffers, including sodium phosphate, triethyl amine-phosphate, triethyl amine-acetate, and triethyl amine-citrate on CE [19]. The results clearly demonstrated that sodium phosphate has the highest S/N ratio. Therefore, it is recommended that sodium or potassium phosphate buffers be utilized for the mobile phase if possible.

3.4.3. Column to column variations

It is known that protein columns tend to have large column-to-column variations in the long term. To minimize variation, it is important to carefully control mobile pH, column temperature, and organic modifier type. We also recommend flushing protein columns after each set of runs: use a pH 2.5 phosphate buffer if the analyte is a basic compound and a pH 6.5 buffer if the analyte is an acidic compound. After this preliminary flushing, follow by flushing with a sufficient amount of water. Interestingly, it was found that flushing the column with a sufficient amount of water before a new set of runs after a long storage period helped to re-condition the column, therefore enhancing the column's reproducibility.

4. Conclusions

The enantioselectivity of 27 drug related compounds including basic, acidic, and neutral molecules have been investigated by RP-HPLC using immobilized protein stationary phases.

AGP and OVM columns demonstrated complimentary enantioselectivity. Greater enantioselectivity was observed for larger molecules on the OVM column and for smaller molecules on the AGP column. Reversal of enantiomer elution order was accomplished by changing stationary phase, organic modifier type, and pH. The enantioselectivity and retention ability of each pair of enantiomers were affected by mobile phase pH, organic modifier type, ionic strength, column temperature, and molecular structure. Various factors are discussed for the development of a valid RPLC chiral method using OVM or AGP column.

Acknowledgment

The authors wish to thank the Process Research Department for providing the studied compounds.

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