Enantioseparation condition of D,L-tryptophan using ligand exchange chromatography

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Chiral recognition mechanism and enantioseparation conditions of tryptophan enantiomers using ligand exchange chromatography are reported here. The effect of different kinds and concentration of ligands, bivalent copper ion, organic modifier, pH of mobile phase, and temperature on enantioseparation has been evaluated. The results show that the enantioselectivity is strongly affected by the pH and the ligand concentration. Under the optimum condition, baseline separation of the two enantiomers has been obtained on a C18 column with a resolution of 3.42 in less than 30 min using methanol-water solution (20:80 v/v) as mobile phase containing 3.0 mmol·L⁻¹ L-phenylalanine and 0.5 mmol·L⁻¹ copper sulphate. Thermodynamic data (ΔΔH and ΔΔS) obtained by Van’t Hoff plots reveal that the enantioseparation is an enthalpy-controlled process. The mechanism of chiral discrimination is based on the stabilities of the copper(II) binary complexes and their ternary diasteremeric complexes with amino acids formed in solution and stationary phase. The proposed method has been successfully used for the quality evaluation of tryptophan enantiomers.

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Amino acids are essential components of many natural molecules and frequently found in food, feeds, body fluids and tissues. Chiral analysis of D,L-amino acids is of great interest in life science and many other related fields. L-tryptophan [L-2-amino-3-(indol-3-yl)propionic acid] is a vital constituent of proteins and indispensable in human nutrition for establishing and maintaining a positive nitrogen balance. As an essential amino acid, L-tryptophan is also considered as the precursor of the neurotransmitter serotonin, which plays an important role in brain function and related regulatory mechanisms. However, D-tryptophan does not show these biological functions and is used as pharmaceutical intermediate.

Enantioseparation of racemic amino acids has been extensively studied by gas chromatography and HPLC using chiral stationary phases or chiral mobile phase additives. Among the chromatographic methods developed thus far, the HPLC methods using chiral stationary phase require a special chiral column, which is expensive and can only separate a couple of enantiomers in most cases. In contrast, HPLC methods based on chiral mobile phase additives (such as β-cyclodextrin or chiral ligand salts) are not only efficient tools for the separation of racemic drugs but also relatively cheap and feasible. Ligand exchange chromatography exploits the rapid and reversible formation of metal ion complexes to separate compounds, which can donate electrons and coordinate to the immobilized metal ions. The enantioselectivity is based on the formation of a kind of ternary complex which conjuncts with an achiral stationary phase to generate an equilibrium-secondary chemical equilibrium. Any difference in the stability or energy of these diasteromeric complexes will affect the chemical equilibrium resulting in different chromatographic behaviour. As a result, the enantiomers can be separated on a conventional HPLC column. Retention of a given species is directly related to the stability of the mixed ligand complex it forms with the metal ion immobilized on a chromatographic support. Ligand exchange with soluble metal complexes, that partition between the mobile and stationary phases, also affects enantioseparation.

We report herein the chiral recognition mechanism and enantioseparation conditions of tryptophan enantiomers on a C18 column using chiral mobile phase additives. The effect of different kinds and concentration of ligands, bivalent copper ion, organic modifier, pH of mobile phase, and temperature on enantioseparation have been evaluated. The chiral recognition mechanisms and thermodynamic characteristic are also discussed.

Experimental

L-phenylalanine, L-serine, L-alanine, L-leucine and L-histidine were purchased from Tokyo Kasei Kogyo Co. LTD (Japan). L-tryptophan and D-tryptophan were obtained from Sigma (ST Louis, MO, USA). Anhydrous cupric sulfate (Extra Pure grade) was purchased from TEDA Company (USA). Acetonitrile, tetrahydrofuran, and methanol were all of HPLC grade and were obtained from Duksan Pure Chemical Co. (Ansan, Korea). Double distilled water...
was filtered with a 0.45 μm filter membrane before use.

**Chiral ligand-exchange RP-HPLC system**

HPLC analysis was performed using a liquid chromatography system containing a Waters 600s Multisolvent Delivery System and a Waters 616 pump (Waters, Milford, MA, USA), a Waters 486 Tunable Absorbance UV detector (Waters, Milford, MA, USA), and a Rheodyne injection valve (20 μL sample loop). Autochro 2000 data software (Younglin Co., Korea) was used as a data acquisition system. The analytical column was packed with C18 stationary phase (OptimaPak 150 mm×4.6 mm I.D., particle size 5 μm, RStech, Daejeon, Korea).

The solution of chiral mobile phase additive (CMPA) comprised of 3.0 mmol L⁻¹ L-phenylalanine mixed with 0.5 mmol L⁻¹ cupric sulfate in water. The mobile phase consisted of CMPA solution-methanol (80:20, v/v). The flow-rate of the mobile phase was set at 1.0 mL min⁻¹. Chromatographic assay was carried out at ambient temperature. UV wavelength was set at 280 nm.

The retention factor was calculated from the equation \( k = (t_R-t_0)/t_0 \), where \( t_R \) and \( t_0 \) are the retention times of analyte and unretained solutes, respectively. The enantioseparation factor was calculated from the equation \( \alpha = k_L/k_D \), where \( k_L \) and \( k_D \) are the retention factors of L-tryptophan and D-tryptophan, respectively. Resolution (\( R \)) was calculated from the equation \( R = 2(t_{L}-t_{D})/(w_D+w_L) \), where \( t_L \) and \( t_D \) are the retention times of L-tryptophan and D-tryptophan, respectively, and \( w_D \) and \( w_L \) are the baseline peak widths of the two enantiomers. The number of theoretical plates (\( N \)) was calculated by the equation \( N=16(t_R/w)^2 \).

**Results and discussion**

**Mechanism of enantioselectivity**

According to the three-point interaction rule of chiral recognition in ligand-exchange chromatography, at least two points of interaction are available for both tryptophan enantiomers: simultaneous coordination of the amine and carboxylate groups to the metal ion. A good selector that discriminates between the two enantiomers should therefore stabilize the third interaction with one of the enantiomers to the maximum possible extent. Chiral selectivity can also be achieved if the third interaction is destabilized for one of the enantiomers relative to the other. Furthermore, the difference in stability of the diastereomeric ternary complexes in aqueous phase has an influence on enantioselectivity and the chiral recognition takes place on the column where the initial complex is adsorbed.

Bivalent copper cation was selected due to the rapid formation and excellent stability of its diastereomeric complexes. First, L-phenylalanine and bivalence metal ion (Cu²⁺) formed a complex, and then incorporated with tryptophan to produce a ternary complex by the carboxylic group and its neighbouring amino group. As tryptophan has two enantiomers, D-tryptophan and L-tryptophan; so, two kinds of ternary complexes with different configurations are formed. The enantioselectivity depends on the differences in the relative stabilities, the energy and the affinity to the stationary phase of cis- and trans- complexes. The complexes formed in mobile phase could conjunct with the stationary phase to generate an equilibrium-secondary chemical equilibrium. Any difference such as stability or energy of these diasteromeric complexes will affect the chemical equilibrium, thereby resulting in their different chromatographic behaviours. As a result, the enantiomers can be separated on a C18 column (Fig. 1).

**Effect of different ligands on enantioselectivity**

An important component of the recognition process is the structure and property of the chiral ligand. In order to investigate the effect of different ligands on the enantioselectivity, L-phenylalanine, L-serine, L-alanine, L-leucine and L-histidine were explored for the enantioseparation of D and L-tryptophan under the same experimental conditions. For amino acids...
with smaller or more flexible side chains as chiral ligand reagent, there was little enantioselectivity. L-phenylalanine (R=3.51) showed better resolution than L-histidine (R=0.81) and L-leucine (R=1.82) (Table 1). No enantioselectivity was observed if L-alanine and L-serine were used as ligand reagents. It appeared that the ligand for enantioseparation should possess a larger group to produce space exclusion function and also should possess certain lipophilicity to be retained by the reverse stationary phase. A consistent trend of higher enantioselectivity with increasing size of the side group was evident, indicating that the size of amino acid side chain is an important factor in determining enantioselectivity in the ligand exchange chromatography.

### Effects of the organic modifier on enantioseparation

The effect of organic modifier on the tryptophan enantioseparation was more significant with less polar organic modifier (acetonitrile and tetrahydrofuran in this case). Tetrahydrofuran as organic modifier hardly showed any enantioselectivity while acetonitrile gave better enantioselectivity (R=0.94) in low flow rate (0.5 mL min⁻¹). The best enantioselectivity was obtained by using methanol as organic modifier. Figure 2 shows that both the retention and separation of the two enantiomers decreased with increasing methanol content. Increasing the organic modifier could decrease the polarity of the mobile phase and consequently diminish the hydrophilic interaction of analytes with the mobile phase. Moreover, the retention ability of L-enantiomers is diminished than D-enantiomers because the lipophilic interaction is more effective in the more stable ternary complex. Although the enantioselectivity increases with a decreased amount of organic modifier, the retention time is longer than 70 min when the concentration of methanol is below 10%. Considering the retention time and enantioselectivity, 20% methanol was selected as the organic modifier for further studies.

### Effects of ligand concentration on enantioseparation

The effect of L-phenylalanine concentration on the enantioselectivity was investigated in a range of 0 to 8.0 mmol L⁻¹ and the results are shown in Fig. 2. With the increase in concentration of L-phenylalanine, the value of k and α increased, and the value of α tended to remain constant when the concentration of ligand was above 3.0 mmol·L⁻¹. Increasing the ligand concentration in the mobile phase could result in the formation of more ligand complexes. These complexes could be distributed on the stationary phase. As a result, the retention and separation of the enantiomers could be increased. When the ligand concentration reached a certain threshold (3.0 mmol L⁻¹), the above parameter tended to remain constant, and the two enantiomers could be baseline separated within 30 min.

### Effects of bivalent copper ion concentration

Different concentrations of Cu²⁺ were investigated in a range from 0 to 6.0 mmol L⁻¹. Figure 2 shows that with the decrease in Cu²⁺ concentration in the mobile phase, both retention and separation of the two enantiomers increased. Additionally, R of the two

<table>
<thead>
<tr>
<th>Different ligands</th>
<th>Retention factor</th>
<th>Selectivity</th>
<th>Resolution</th>
<th>Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>k_D k_L</td>
<td>α</td>
<td>(R)</td>
<td>N_D N_L</td>
</tr>
<tr>
<td>L-phenylalanine</td>
<td>19.43 23.99</td>
<td>1.23</td>
<td>3.51</td>
<td>4799 5011</td>
</tr>
<tr>
<td>L-serine</td>
<td>6.38 6.38</td>
<td>1.00</td>
<td>0.00</td>
<td>2464 2464</td>
</tr>
<tr>
<td>L-alanine</td>
<td>6.77 6.77</td>
<td>1.00</td>
<td>0.00</td>
<td>1089 1089</td>
</tr>
<tr>
<td>L-histidine</td>
<td>5.95 6.48</td>
<td>1.09</td>
<td>0.81</td>
<td>2740 1418</td>
</tr>
<tr>
<td>L-leucine</td>
<td>13.45 15.08</td>
<td>1.12</td>
<td>1.82</td>
<td>4637 4736</td>
</tr>
</tbody>
</table>
enantiomers increased from 1.81 to 3.53 when Cu$^{2+}$ concentration decreased from 6.0 to 0.3 mmol L$^{-1}$. Although decrease the Cu$^{2+}$ concentration in mobile phase could increase the retention and separation of both D- and L-tryptophan continuously, the elution time of tryptophan enantiomers was longer than 30 min if the concentration of Cu$^{2+}$ was lower than 0.5 mmol L$^{-1}$. Considering the retention and separation, 0.5 mmol L$^{-1}$ was used as the optimum Cu$^{2+}$ concentration.

**Effects of pH of the mobile phase**

pH-dependence of the enantioselectivity was investigated in a pH range 4.1 to 5.2 using phosphoric acid, hydrochloric acid, and trifluoroacetic acid to adjust pH. In contrast to reports that changing the pH of the mobile phase hardly influenced the resolution$^{20}$, our results showed that the resolution of tryptophan enantiomers distinctly decreased from 3.42 to 0 when the pH of the mobile phase was lowered from 4.9 to 4.1. Also, no enantioselectivity was observed at lower pH, which was due to the increased acid in mobile phase destroying the stereo structures of the ternary diasteremeric complex. When the pH of the mobile phase exceeds 5.0, Cu$^{2+}$ precipitates and blocks the chromatographic systems. Hence, 4.9 were chosen as the optimum pH of the mobile phase.

**Effect of temperature on the enantioseparation**

The temperature-dependence of the enantioseparation was investigated in the range 20°C to 50°C. At lower temperature, the formation of the two transient diastereomeric ternary complexes is expected to be more favourable. Consequently, retention times improve at lower temperature. In addition, the formation of more stable ternary complex is expected to be more favorable than that of the less stable ternary complex at lower temperature. The difference in the stability of the two diastereomeric ternary complexes increases and the separation factors increase as the temperature decreases. However, $R$ only slightly changes from 3.42 to 3.36 while temperature increases from 20°C to 50°C, which is due to the rate of equilibrium for the formation of ternary complexes is slow at lower temperature. Thus, the lifetime of the transient diastereomeric ternary complex is quite long at lower temperature and, consequently, the chromatographic peaks corresponding to the two enantiomers broaden.

**Thermodynamics of enantioseparation**

The retention behaviour and thermodynamic parameters determined in this study were used to estimate the enthalpy, entropy, and Gibbs free energy of association between the two enantiomers and the C$_{18}$ stationary phase. Data obtained from retention and separations at temperature ranging from 20°C to 50°C were processed using the Van’t Hoff equation to estimate the thermodynamic properties of the separation:

$$
\ln k = -\frac{\Delta G}{RT} = -\frac{\Delta H}{RT} + \frac{\Delta S}{R}
$$
\[ \Delta G = -\frac{\Delta H}{RT} - \frac{\Delta S}{R} \]

where \( R \) and \( T \) are the gas constant and the absolute temperature (Fig. 3). Enthalpy (\( H \)) and entropy (\( S \)), enthalpy difference (\( H \)) and entropy difference (\( S \)) can be calculated from the slopes and intercepts of linear portion of above equation. The results are listed in Table 2.

The absolute values of \( H \) and \( S \) of L-tryptophan were larger than D-tryptophan indicating that L-tryptophan has stronger affinity to the stationary phase during the separation. Moreover, the finding that \( |H| > |S| \) indicates that the chiral separation on the C\(_{18}\) column is an enthalpy-controlled process. In general, for the temperature range 20-50\( ^\circ \)C. The enthalpic contribution to the overall substrates transfer energy of the substrates was found to be more significant than the entropic contribution. The decrease in temperature led to an increase in the separation factors, which is consistent with the results of the temperature-dependence studies.

### Quantitative determination

In order to assure the reliability of the assay, calibration graphs were constructed by using the areas of the chromatographic peaks measured at eight increasing concentrations in the range 1.0 to 200 mg L\(^{-1}\) for the both tryptophan enantiomers. Good linearity throughout the concentration is obtained for both enantiomers and the linear correlation equations were:

- For D-tryptophan: \( Y= 84.73x+172.84 \)
- For L-tryptophan: \( Y=81.66x+165.32 \)

with coefficient of regression (\( r \)) more than 0.999, respectively. Precision and accuracy were assessed by performing replicate analyses of quality control samples against calibration standards and were calculated as the relative standard deviation (RSD). The procedure was repeated on the same day and four different days on the same spiked standards at concentrations in the range of standard series. The intra-assay relative standard deviations and inter-assay relative standard deviations of the proposed method, determined on the basis of peak-area for three replications, were lower than 0.67% and 1.21% for D-tryptophan and 0.59% and 1.86 % for L-tryptophan, respectively.

### Conclusions

The proposed method can be used for the quality evaluation of tryptophan enantiomers. The results show that the enantioselectivity is strongly affected by the \( pH \) of mobile phase and the ligand concentration. The selectivity of tryptophan enantioseparation depends on the stabilities of the ternary diasteremeric complexes. Baseline separation of the two enantiomers with a resolution of 3.42 in less than 30 min can be obtained on the C\(_{18}\) column. Thermodynamic data (\( H \) and \( S \)) obtained by Van’t Hoff plots reveal that the enantioseparation is an enthalpy-controlled process.

### Acknowledgement

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### Table 2—Thermodynamic parameters of tryptophan enantioseparation

<table>
<thead>
<tr>
<th>Analytes</th>
<th>( \Delta H ) (J.mol(^{-1}))</th>
<th>( \Delta S ) (J.mol(^{-1}).K(^{-1}))</th>
<th>( \Delta\Delta H ) (J.mol(^{-1}))</th>
<th>( \Delta\Delta S ) (J.mol(^{-1}).K(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-tryptophan</td>
<td>-6956</td>
<td>-4.222</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>L-tryptophan</td>
<td>-7653</td>
<td>-4.574</td>
<td>-693.8</td>
<td>-0.2619</td>
</tr>
</tbody>
</table>

\( \Delta H \) and \( \Delta S \) were obtained from linear regression of the Van’t Hoff plots by plotting \( \ln k' \) versus \( 1/T \); \( \Delta\Delta H \) and \( \Delta\Delta S \) were obtained from plot of \( \ln a \) versus \( 1/T \).

### References