

Original

# Thermodynamic Properties of Enantioseparation of Two *N*-Carbobenzyloxy-*D*, *L*-Amino Acids on Cellulose Tris (4-methylbenzoate) Chiral Stationary Phase

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## Abstract

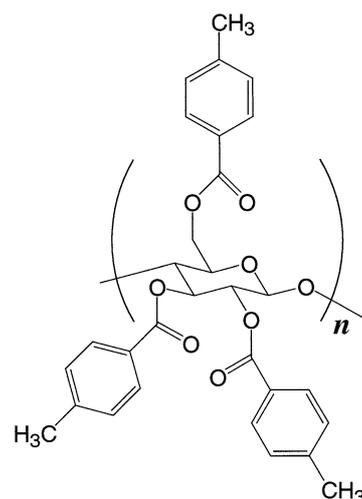
The isocratic retention of enantiomers of *N*-carbobenzyloxy-*D*, *L*-tryptophan and *N*-carbobenzyloxy-*D*, *L*-phenylalanine was studied on cellulose tris (4-methylbenzoate) coated on silica as a chiral stationary phase at different temperatures and with different mobile phase compositions in the reversed-phase mode. The thermodynamic parameters were calculated in order to promote an understanding of the relationship between enthalpy and entropy for enantioseparation in this chromatographic system. The separation was enthalpy-controlled with negative entropic contribution in the HPLC condition studied. The enthalpy-entropy relationship indicates that enthalpic gain/loss for enantioseparation from any change of organic modifier content in the mobile phase is almost cancelled out by the entropic loss/gain.

**Keywords:** cellulose tris (4-methylbenzoate) chiral stationary phase, enantioseparation, enthalpy-entropy compensation

## 1. Introduction

The separation and analysis of biologically active chiral compounds continue to be an area of increasing interest in pharmaceutical industry since biological activities may vary dramatically between enantiomers. Through the advances of industrial technologies, direct chiral high-performance liquid chromatographic separation is now well established with many commercially available columns containing chiral stationary phases (CSPs). In the past few years, cellulose-based derivatives have been developed and shown to be exceptionally useful for the separation of enantiomers of biological and pharmaceutical importance. Cellulose tris (4-methylbenzoate) is one of the most recently introduced CSPs (Figure 1).

Although there is now a considerable amount of information regarding interaction between solutes and cellulose-based chiral stationary phases, [1-3] little is known from a view of thermodynamics, especially in the reversed-phase mode. The degree of chiral recognition of enantiomers by a CSP depends strongly on the temperature. The separation factor usually decreases as the temperature increases. Retention data obtained at different tempera-



**Figure 1.** Chemical structure of cellulose tris (4-methylbenzoate).

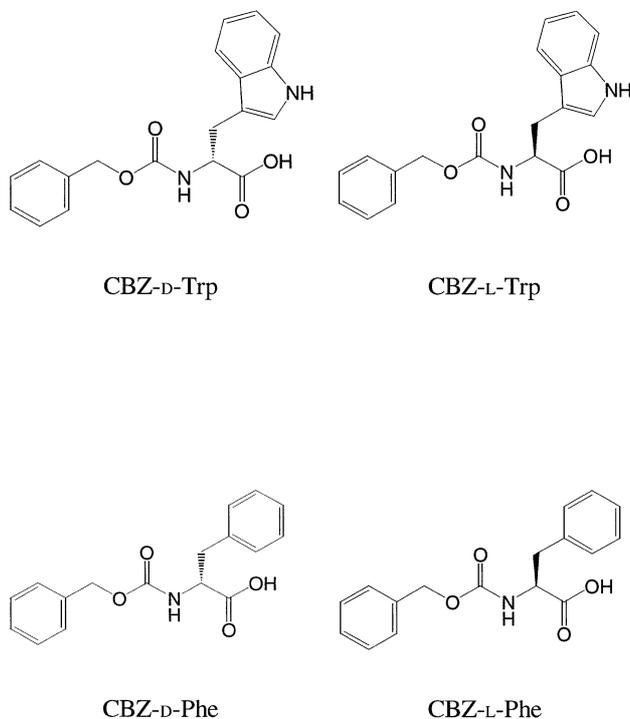
tures allow an evaluation of the variations in enthalpy and entropy, which govern the free energy of transfer of the analyte from the mobile phase to the stationary phase. These thermodynamic pa-

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rameters were determined in order to better understand the thermodynamic driving forces affecting retention and separation of enantiomers. These effects on retention in reversed-phase high-performance liquid chromatography have been considered for different CSPs, including those based on chiral crown ethers, [4, 5] cyclodextrins, [6, 7]  $\alpha$ -acid glycoprotein, [8] human serum albumin, [9] derived aromatic ring, [10–12] derived amylose [13, 14] and cellulose, [14–19] and chiral macro cyclic antibiotics [20] by many groups.

Our recent studies, from the thermodynamic point of view, revealed the conformation change of the CSP consisted of amylose tris (3, 5-dimethylphenylcarbamate) over the temperature range studied, [13] and unusual behavior of the enthalpy and entropy of transfer of the solute from the mobile to the CSP of cellulose tris (3, 5-dimethylphenylcarbamate) as a function of organic modifier content in the mobile phase, [18] respectively. The aim of the present report is to provide the information concerning the relation between enthalpy and entropy on the mechanisms involved in the separation of derived D, L-amino acids with cellulose tris (4-methylbenzoate) coated on silica as a chiral stationary phase in the reversed-phase mode.



**Figure 2.** Chemical structures of derived amino acids:  
*N*-carbobenzyloxy-D-tryptophan (CBZ-D-Trp),  
*N*-carbobenzyloxy-L-tryptophan (CBZ-L-Trp),  
*N*-carbobenzyloxy-D-phenylalanine (CBZ-D-Phe),  
*N*-carbobenzyloxy-L-phenylalanine (CBZ-L-Phe).

## 2. Experimental

### 2.1. Chemicals and reagents

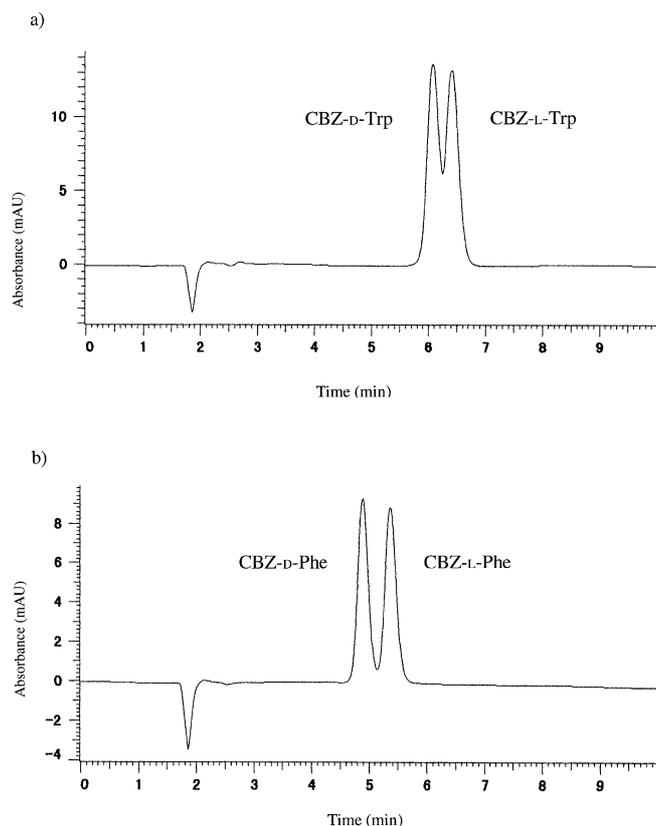
Acetonitrile and methanol of HPLC grade, phosphoric acid and sodium dihydrogen phosphate were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Water was deionized prior to usage. Sodium dihydrogen phosphate was dissolved in deionized water and phosphoric acid was added to make a 0.02-M phosphate buffer at pH 2.0. The amino acid derivatives investigated were *N*-carbobenzyloxy-D, L-phenylalanine (CBZ-D, L-Phe), *N*-carbobenzyloxy-L-phenylalanine (CBZ-L-Phe), *N*-carbobenzyloxy-D, L-tryptophan (CBZ-D, L-Trp) and *N*-carbobenzyloxy-L-tryptophan (CBZ-L-Trp) (Figure 2) from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Derived amino acids were dissolved in acetonitrile at 0.01 mg/mL for chiral HPLC analysis. The L-enantiomers were employed for the identification of elution order of enantiomers.

### 2.2. Apparatus

HPLC measurements were performed on a Hitachi (Tokyo, Japan) D-7000 System equipped with two isocratic pumps (L-7100), variable wavelength detector (L-7420), variable volume injector (L-7200) and column oven (L-7300). CHIRALCEL OJ-RH (150 mm × 4.6 mm I.D., Daicel Chemical Ltd., Tokyo, Japan), kindly provided from Fuji Kagaku Yakuhin (Hyogo, Japan), was employed for analytical separation. The packing material of the column was a silica-gel support (5  $\mu$ m) coated with a polymer of cellulose tris(4-methylbenzoate). The mobile phase consisted of acetonitrile – 0.02 M phosphate buffer at pH 2.0 (33:67–41:59, v/v). The column temperature was adjusted at 20–45 °C at a flow-rate of 1 mL/min, and the injection volume was set at 10  $\mu$ L. Duplicate injections were performed at each column temperature and mobile phase composition. The elution of amino acid derivatives was monitored at 220 nm. The chromatographic system was equilibrated by passing the eluent until a stable baseline signal was obtained. This procedure was always followed when a new mobile phase or temperature was applied for analysis. The column void time was determined from the first perturbation of baseline caused by methanol injection.

## 3. Results and discussion

Enantiomers of CBZ-D, L-Trp and CBZ-D, L-Phe were chromatographed on CHIRALCEL OJ-RH in the reversed-phase mode. Acetonitrile was used as organic modifier due to the poor durability of CHIRALCEL OJ-RH for pressure. Typical chromatograms are shown in Figure 3. As regards the elution order of enantiomers, the D-enantiomers always eluted before the L-enantiomers. In some cases the organic modifier in the mobile phase plays an important role in the stereo-selectivity by binding to



**Figure 3.** Chromatograms of amino acid derivatives. a) : CBZ-D, L-Trp, b) : CBZ-D, L-Phe. Column, CHIRALCEL OJ-RH; mobile phase, acetonitrile-phosphate buffer (40:60, v/v); column temperature, 45 °C; detection, 220 nm; flow-rate, 1 mL/min.

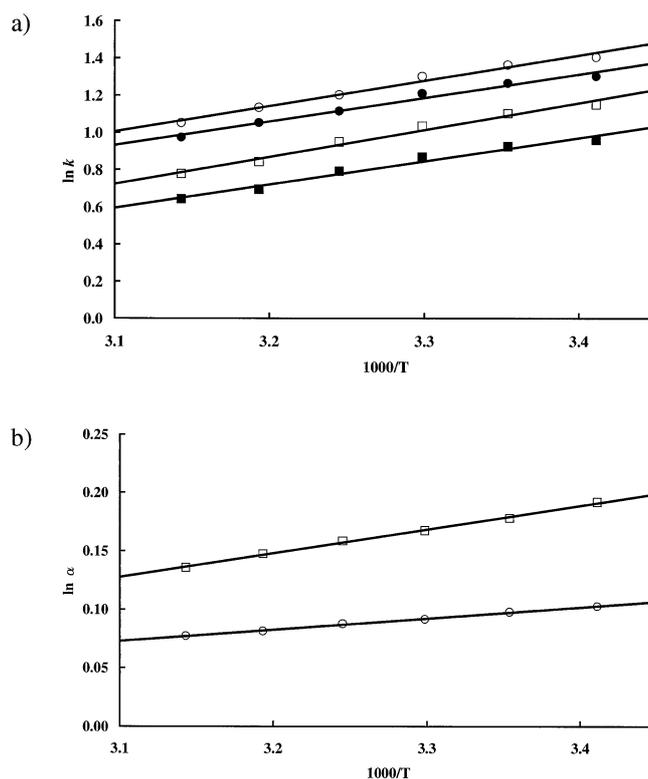
achiral sites near or at the chiral cavities of CSP, which alters the steric environment of these cavities. [17] However, the chiral separation on this CSP was not affected by varying acetonitrile content in the range tested.

In order to calculate thermodynamic parameters for more understanding of the separation mechanism of enantiomers on this CSP, van't Hoff plots were constructed. The chromatographic retention, expressed by  $k$ , was expressed in terms of the following van't Hoff equation:

$$\ln k = -\Delta H^\circ/RT + \Delta S^\circ/R + \ln V_R \quad (1)$$

In this equation,  $\Delta H^\circ$  and  $\Delta S^\circ$  are enthalpy and entropy changes of the transfer from the mobile to the stationary phase.  $R$  is the gas constant, and  $T$  is the absolute temperature of the chromatographic system.  $V_R$  means the phase ratio of the column (the volume of the stationary phase divided by the volume of the mobile phase). This expression shows that the plot of  $\ln k$  versus  $1/T$  should be linear if  $\Delta H^\circ$ ,  $\Delta S^\circ$  and  $V_R$  are invariant with temperature in the range tested. Furthermore,

$$\begin{aligned} \ln \alpha &= \ln (k_L/k_D) \\ &= -\Delta\Delta H^\circ/RT + \Delta\Delta S^\circ/R \end{aligned} \quad (2)$$

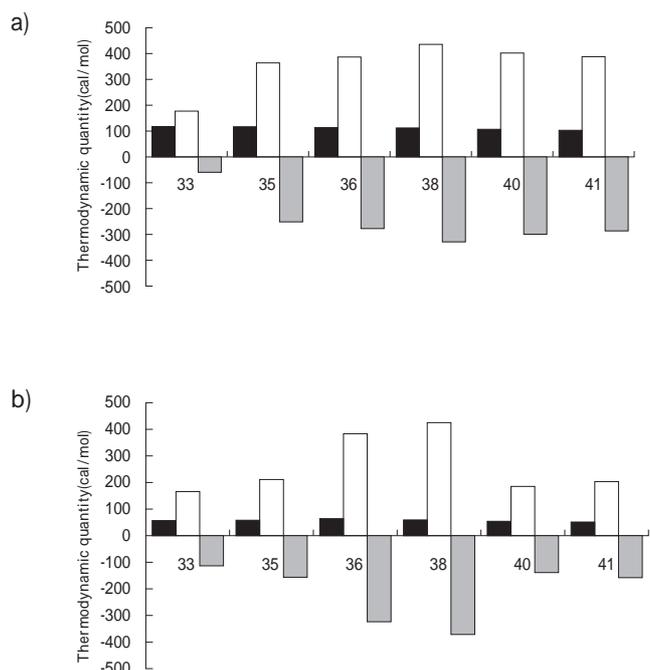


**Figure 4.** Temperature dependence of retention and enantio-separation of derived amino acids on a CSP. Column, CHIRALCEL OJ-RH; mobile phase, acetonitrile-phosphate buffer (40:60); detection, 220 nm; flow-rate, 1 mL/min. a) van't Hoff plots for retention :  $\bullet$ , CBZ-L-Trp ;  $\circ$ , CBZ-D-Trp ;  $\blacksquare$ , CBZ-L-Phe ;  $\square$ , CBZ-D-Phe. b) van't Hoff plot for Enantio-separation :  $\bullet$ , CBZ-D, L-Trp ;  $\square$ , CBZ-D, L-Phe.

where  $k_L$  and  $k_D$  are the capacity factors of L- and D-enantiomers.  $\Delta\Delta H^\circ$  and  $\Delta\Delta S^\circ$  represent the differences of the enthalpy and entropy changes of the enantiomers, respectively.  $\ln \alpha$  versus  $1/T$  (van't Hoff plot of  $\alpha$ ) should also be linear if  $\Delta\Delta H^\circ$  and  $\Delta\Delta S^\circ$  are invariant with temperature in the range tested. Linear and non-linear van't Hoff plots were reported in the temperature studies in the reversed-phase mode. [13, 18, 21–24] Non-linear van't Hoff plots may be indicative of a change in the mechanism of retention.

Figure 4 illustrates the representative linear van't Hoff plots of  $k$  and  $\alpha$  with correlation coefficients both of 0.999 for derived amino acids employing the mobile phase, acetonitrile-phosphate buffer of 40:60 (v/v), respectively. These graphs indicate that the mechanisms for retention and separation of phenylalanine- and tryptophan-derivatives on the CSP were not changed in the temperature range studied.

The enthalpic and entropic contributions to the chiral separation of D- and L-enantiomers of derived amino acids are visualized



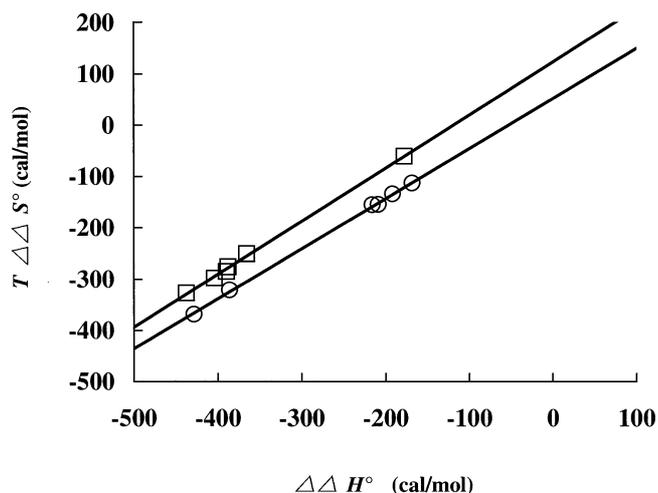
**Figure 5.** Differences in free energy, enthalpy and entropy for chiral separation of derived amino acids on cellulose tris(4-methylbenzoate) column at 25 °C using the mobile phase of acetonitrile – phosphate buffer. The numbers on the abscissa indicate acetonitrile content in the mobile phase. Filled, unfilled and shaded parts indicate  $-\Delta\Delta G^\circ$ ;  $-\Delta\Delta H^\circ$  and  $T\Delta\Delta S^\circ$ , respectively. a) CBZ-D, L-Phe; b) CBZ-D, L-Trp.

in Figure 5. On the basis of thermodynamic data, Figure 5 clearly shows that the chiral separation of CBZ-D, L-Trp and CBZ-D, L-Phe on cellulose tris (4-methylbenzoate) column are exclusively dominated by enthalpy with negative entropic contribution. With an increase of acetonitrile content in the mobile phase, the  $-\Delta\Delta H^\circ$  values increased, reached maximum values at about 38% of acetonitrile, and decreased. This trend was cancelled out by the  $T\Delta\Delta S^\circ$  values, resulting in the constant  $-\Delta\Delta G^\circ$  values. This is the reason for constant separation factors for respective racemates.

Using these data sets for chiral separation, the differences of entropy are plotted against the differences of enthalpy to generate straight lines with correlation coefficients of more than 0.999, as shown in Figure 6. As the magnitude of the enthalpy for enantio-separation decreases, an offsetting increase in the entropy occurs. A relationship of this type is referred to as enthalpy – entropy compensation. [25, 26] For these straight lines the resulting change in entropic term is proportional to the accompanying change in enthalpy as described in the following equation 3:

$$T\Delta(\Delta S^\circ) = \Delta(\Delta H^\circ) \quad (3)$$

where  $\Delta$  means the slope of this straight line. The slope ( $\Delta$ ) is a quantitative measure of the entropic canceling of the enthalpic gain



**Figure 6.** Enthalpy–entropy compensation plots for chiral recognition of enantiomers of CBZ-D, L-Trp and CBZ-D, L-Phe.  $\square$ , CBZ-D, L-Trp;  $\circ$ , CBZ-D, L-Phe.

for enantio-separation. Integration of equation 3 gives equation 4, where  $T\Delta\Delta S_0$  refers to the value at  $\Delta\Delta H^\circ = 0$  (intercept) of Figure 6.

$$T\Delta\Delta S^\circ = \Delta\Delta H^\circ + T\Delta\Delta S_0 \quad (4)$$

Enantiomers are solvated identically in the mobile phase, but would release a different number of solvent molecules when they associate with the CSP. Therefore, this contribution to entropy would not be identical for both enantiomers. Since  $\Delta\Delta S^\circ$  values are negative, L-enantiomers have fewer degrees of freedom on the CSP. In other words, L-enantiomers are held at more points or are less able to move or rotate in the CSP. Figure 6 indicates that as far as the intercept of the ordinate is positive, that is indeed the case of CBZ-D, L-Phe and CBZ-D, L-Trp with this CSP under the HPLC condition studied, the enantio-separation can take place even in the absence of enthalpic gain ( $-\Delta\Delta H^\circ$ ). This result suggests that the enantio-separation without enthalpic contribution would be achieved by the difference in desolvation from each enantiomer adsorbed through achiral contribution in the chiral cavity.

Differential form of Gibbs free energy function for interaction of an enantiomeric pair with the CSP is the following:

$$\Delta(\Delta G^\circ) = \Delta(\Delta H^\circ) - T\Delta(\Delta S^\circ) \quad (5)$$

Inserting equation 3 in equation 5, equation 6 is obtained.

$$\Delta(\Delta G^\circ) = (1 - \Delta) \Delta(\Delta H^\circ) \quad (6)$$

Equation 6 indicates that only  $(1 - \Delta)$  proportion of the increment in enthalpy ( $-\Delta\Delta H^\circ$ ) contributes toward raising the enantio-separation ( $-\Delta\Delta G^\circ$ ).

As can be seen from Figure 6, both linear plots give large slopes ( $\Delta$ : 0.98 for CBZ-D, L-Trp, 1.03 for CBZ-D, L-Phe). These slopes (approximately 1) indicate that the enthalpic gain or loss arising from any change caused by mobile phase composition is

not reflected on the chiral separation on cellulose tris (4-methylbenzoate) CSP in this chromatographic system. In brief,  $\Delta\Delta G^\circ$  is kept constant by the compensation of  $\Delta\Delta H$  and  $T\Delta\Delta S^\circ$  under the HPLC condition studied.

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