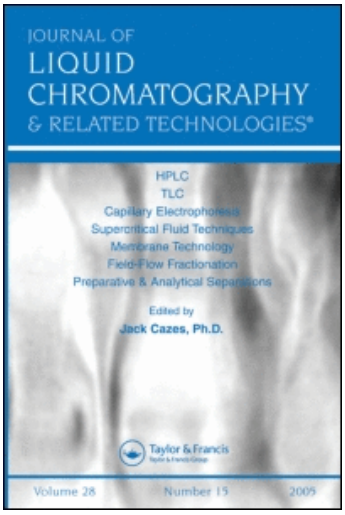

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

A CHROMATOGRAPHIC METHOD FOR MEASURING K_F OF ENANTIOMER-CHIRAL SELECTOR COMPLEXES

Ying Ma[a]; Yoichiro Ito[a]; Alain Berthod[b]
[a] Laboratory of Biophysical Chemistry, 10 Center Drive, MSC 1676, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, U.S.A. [b] Université de Lyon 1, Villeurbanne Cedex, France

Online publication date: 17 November 1999

To cite this Article Ma, Ying , Ito, Yoichiro and Berthod, Alain(1999) 'A CHROMATOGRAPHIC METHOD FOR MEASURING K_F OF ENANTIOMER-CHIRAL SELECTOR COMPLEXES', Journal of Liquid Chromatography & Related Technologies, 22: 19, 2945 — 2955
To link to this Article: DOI: 10.1081/JLC-100102070
URL: http://dx.doi.org/10.1081/JLC-100102070

J. LIQ. CHROM. & REL. TECHNOL., 22(19), 2945–2955 (1999)

A CHROMATOGRAPHIC METHOD FOR MEASURING K_F OF ENANTIOMER-CHIRAL SELECTOR COMPLEXES

Ying Ma,[1] Yoichiro Ito,[1,*] Alain Berthod[2]

[1] Laboratory of Biophysical Chemistry
National Heart, Lung, and Blood Institute
National Institutes of Health
10 Center Drive, MSC 1676,
Bethesda, MD 20892-1676, USA

[2] Laboratoire des Sciences Analytiques
Université de Lyon 1
Batiment 308
69622 Villeurbanne Cedex, France

ABSTRACT

The method uses high-speed countercurrent chromatography to retain a given concentration of the chiral selector (CS) in the liquid stationary phase. A minute amount of an enantiomeric analyte is eluted through the column to measure its retention time from which the distribution ratio of the analyte is computed. The experiment is repeated by varying the CS concentration in the stationary phase. Using a set of data thus obtained, plotting the CS concentration in the stationary phase against the relative distribution ratio of the analyte produces a straight line whose slope corresponds to the formation constant (K_f) of the CS-analyte complex. The validity of the method is demonstrated on a set of dinitrobenzoyl amino acids using N-dodecanoyl-L-proline-3,5-dimethylanilide as CS. The method will be useful for understanding the basic mechanism of enantioselectivity and designing effective chiral selectors.

INTRODUCTION

Over the past years, the important role of chirality in biochemistry and drug design has been increasingly recognized and extensive efforts have been made to develop methods for resolving racemic mixtures. At present chiral separations in the laboratory are mostly performed by liquid chromatography using chiral columns where chiral selector molecules are permanently bound to the solid support.

Despite rather intensive studies on enantioselectivity conducted by Pirkle et al.,[1] the selection of suitable chiral selectors for target enantiomers still relies on serial trial runs using multiple chiral columns in most cases.

The parameter describing the enantioselectivity is the formation constant (K_f) of the enantiomeric analyte-chiral selector complex. However, this parameter is not easily obtained by conventional liquid chromatographic techniques which use chiral selectors immobilized onto the matrix thus preventing the determination of their concentrations.

Shibukawa et al.[2] recently reported a method for measuring K_fs of enantiomer-cyclodextrin complexes using capillary electrophoresis. However, their technique requires correction factors because of the viscosity of the medium due to its high concentration of chiral selector. These factors are tedious to determine and result in diminished accuracy. In addition, the analyte is required to be ionic, and both analyte and chiral selector must have adequate solubility in an aqueous solution.

Here we report a simple and reliable method of measuring formation constants (K_f) for the chiral selector-enantiomer complexes. This information is critical in understanding the basic mechanism responsible for enantioselectivity, which is necessary for the design of more effective chiral selectors.

EXPERIMENTAL

Apparatus

A multi-layer coil planet centrifuge (P.C. Inc., Potomac, MD, USA) was used for performing high-speed CCC separation. It holds a separation column at 10 cm from the central axis of the centrifuge while a counter-weight was mounted on the other side for balancing the centrifuge. The multi-layer coil separation column consisted of a 1.6 mm I.D., 130 m long PTFE (polytetrafluoroethylene) (Zeus Industrial Products, Raritan, NJ, USA) tubing with a total capacity of 315 mL. The β value of the column measures 0.5 to 0.75. The column was rotated at 800 rpm with a speed control unit.

Reagents

Organic solvents including hexane, ethyl acetate, methanol, were glass distilled grade (Burdick & Jackson Labs. (Muskegon, MI, USA). Chiral selector, N-dodecanoyl-L-proline-3,5-dimethyl-anilide, was synthesized according to the method described by Oliveros et al.[3] Various kinds of dinitrobenzoyl(DNB)-amino acids were all purchased from Aldrich (Milwaukee, WI, USA).

THEORY

Our method for measuring the K_f value described below uses high-speed countercurrent chromatography (CCC) which eliminates the use of solid support.[4-6] In this technique, the separation of enantiomers can be carried out by dissolving the chiral selector in the stationary liquid phase.[7,8] This enables one to determine an accurate molar concentration of the chiral selector in the column, an essential number for determination of the formation constant.

The experimental procedure used in the present studies is as follows: A two-phase solvent system is equilibrated in a separatory funnel and the two phases separated. The chiral selector (CS) is dissolved in the stationary phase at a given molar concentration, $[CS]_{initial}$. In each separation, the column is first filled with the stationary phase followed by injection of the sample solution containing a minute amount of the target racemate. The column is then eluted with the mobile phase while the apparatus is rotated at a desired speed. The effluent from the outlet of the column is continuously monitored with a UV monitor. From the chromatogram thus obtained, the distribution ratio ($D_\pm$) of enantiomers can be calculated according to the conventional equation:[6]

$$D_\pm = (V_\pm - V_m)/(V_c - V_m) \quad (1)$$

where $V_\pm$ is retention volumes of the enantiomers; V_m, the volume of the mobile phase in the column (or the retention volume of the solvent front); and V_c, the total column capacity. Using the stationary phase free of CS under otherwise identical experimental conditions, the partition ratio (D_0) of the enantiomer is obtained according to the following equation:

$$D_0 = (V_0 - V_m)/(V_c - V_m) \quad (2)$$

where V_0 is the retention volume of the enantiomer. Both $D_\pm$ and D_0 values thus obtained are used for computing the formation constant as shown below.

Figure. 1 schematically shows a cross-sectional view through the separation column where two phases are arbitrarily separated, the organic stationary phase containing the CS in the upper portion and the aqueous mobile phase in the lower portion.

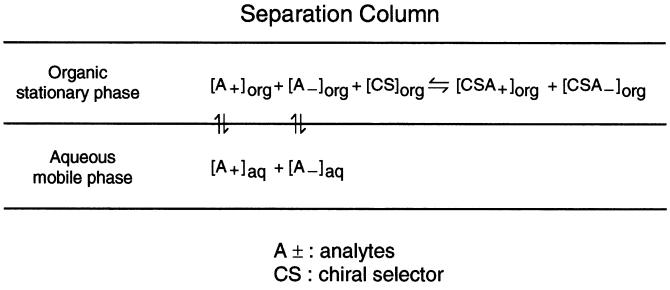

Figure 1. Dynamic equilibrium of chiral selector (CS) and enantiomers in the column of a high-speed countercurrent chromatograph.

The enantiomeric analytes, $A_{\pm}$, are distributed between the two phases at the distribution ratios ($D_{\pm}$) and in the upper organic phase they form complexes with the CS according to their formation constants, $K_{f\pm}$ where the concentration of K_f in the aqueous phase is negligible.

The relationship between these parameters is expressed by the following equations:

$$D_{\pm} = ([A_{\pm}]_{org} + [CSA_{\pm}]_{org})/[A_{\pm}]_{aq} \quad (3)$$

$$D_0 = [A_{\pm}]_{org}/[A_{\pm}]_{aq} \quad (4)$$

$$K_{f\pm} = [CSA_{\pm}]_{org}/[A_{\pm}]_{org}\,[CS]_{org} \quad (5)$$

From Eqs. 3 - 5,

$$D_{\pm} = D_0\,(1 + K_{f\pm}[CS]_{org}) \quad (6)$$

When the concentration of analytes, $[A_{\pm}]_{org}$ is much smaller than that of the CS in the organic phase, $[CS]_{org}$, Eq. 6 may be approximated as

$$D_{\pm} = D_0\,(1 + K_{f\pm}[CS]_{initial}) \quad (7)$$

where $[CS]_{initial}$ indicates the CS concentration initially introduced into the organic stationary phase. Since $D_{\pm}$, D_0 and $[CS]_{initial}$ are known, $K_{f\pm}$ can be determined from Eq. 7.

When $D_{\pm}$ values are plotted against various values of $[CS]_{initial}$ in abscissa, data should form a straight line which intersects the ordinate at D_0 while the slope indicates $K_{f\pm}D_0$ from which $K_{f\pm}$ can be obtained.

Table 1

Parameters of the K Versus $[CS]_{Initial}$ Lines

Enantiomer	Form	Slope	Intercept	r	D_0	(D_0')	K_f (L mol^{-1})	$\Delta(\Delta G0_c)$ (kJ mol^{-1})
DNB-phenylglycine	-	6.1	0.150	0.963	0.155	(0.146)	39	1.32
	+	17.3	0.159	0.983	0.155	(0.146)	112	
DNB-phenylalanine	-	7.4	0.193	0.924	0.190	(0.190)	38	1.91
	+	26.3	0.188	0.984	0.190	(0.190)	140	
DNB-valine	-	4.7	0.142	0.996	0.146	(0.146)	32	1.96
	+	18.6	0.149	0.999	0.146	(0.146)	131	
DNB-leucine	-	9.9	0.284	0.999	0.285	(0.280)	35	3.16
	+	71.8	0.285	0.998	0.285	(0.280)	252	

CS: chiral slector: N-dodecanoyl-L-proline-3,5-dimethylanilide; DNB: 3,5-dinitrobenzoyl; D_0: partition ratio of the enantiomer obtained by linear regression analysis; D_0': partition ratio of the enantiomer obtained by experiment; K_f: formation constant of the CS-enantiomer complex; $\Delta(\Delta G_c)$: difference in the molecular free energy of the chiral interaction between the + (L) and - (D) enantiomers with 0.02 M (8 g/L) CS concentration.

RESULTS AND DISCUSSION

The validity of this approach has been examined by a series of experiments where small amounts (0.1 mg to 1 mg each) of (±)-DNB(dinitrobenzoyl) amino acids were separated using various concentrations of the chiral selector (N-dodecanoyl-L-proline-3,5-dimethylanilide) at 0.5 g, 1 g, 2g, and 4 g each dissolved in 200 mL of the organic stationary phase. An acidic solvent system composed of hexane/ethyl acetate/methanol/10mM HCl (8:2:5:5, v/v) was used to cause protonation of analyte that was in the aqueous phase. As expected, the experimental $D_{\pm}$ values plotted against the initial CS concentrations showed a straight line for each enantiomer where the intercept to the ordinate was almost identical to the corresponding D_0 value. The slope of the straight line corresponded to the product $K_f D_0$ from which the formation constant, K_f, is obtained (Table 1).

For each pair of racemates examined, the average value of the intercepts (D_0) closely matched the partition ratio (D_0' in Table 1) directly obtained from the experiment using a CS-free solvent system. These results indicate that Eq. 7 is useful for computing the formation constant of various analyte-CS pairs.

Practically, it is more convenient to modify Eq. 7 so that the slope directly indicates the formation constant, i.e.,

$$(D_{\pm} - D_0)/D_0 = [CS]_{initial} K_f \qquad (8)$$

Figure. 2 shows the K_f values of (±)-DNB amino acids having various lengths of hydrocarbon chains ranging from 2 (DNB-alpha-amino butyric acid) to 8 (DNB-capric acid) at the asymmetric carbon site. The formation constants of D_- enantiomers all lie in a narrow range between 19 and 51 while those of D_+ enantiomers are much greater and vary in a broad range from 100 to 250 somewhat correlating with the length of the hydrocarbon chain at the site of the asymmetric carbon in both aliphatic and aromatic groups. In D_+ aliphatic enantiomers, the K_f value increases with the length of the hydrocarbon chain from 2 (DNB-alanine) to 4 (DNB-leucine) which shows the greatest value (250). Further increasing the chain length to 6 (capric acid) results in decreasing the K_f value.

The K_f values can be used to study the chiral recognition mechanism. Eqs. 6 and 7 show that the enantiomers are retained in the stationary phase by hydrophobic effect (the D_0 distribution ratio) and by chiral interactions (the K_f formation constant). The two enantiomers have the same D_0 value. For a polarity point of view, they interact identically with the organic stationary phase. The enantioselectivity factor, α, is expressed as the ratio of the experimental distribution factors:

$$\alpha = D_+/D_- \qquad (9)$$

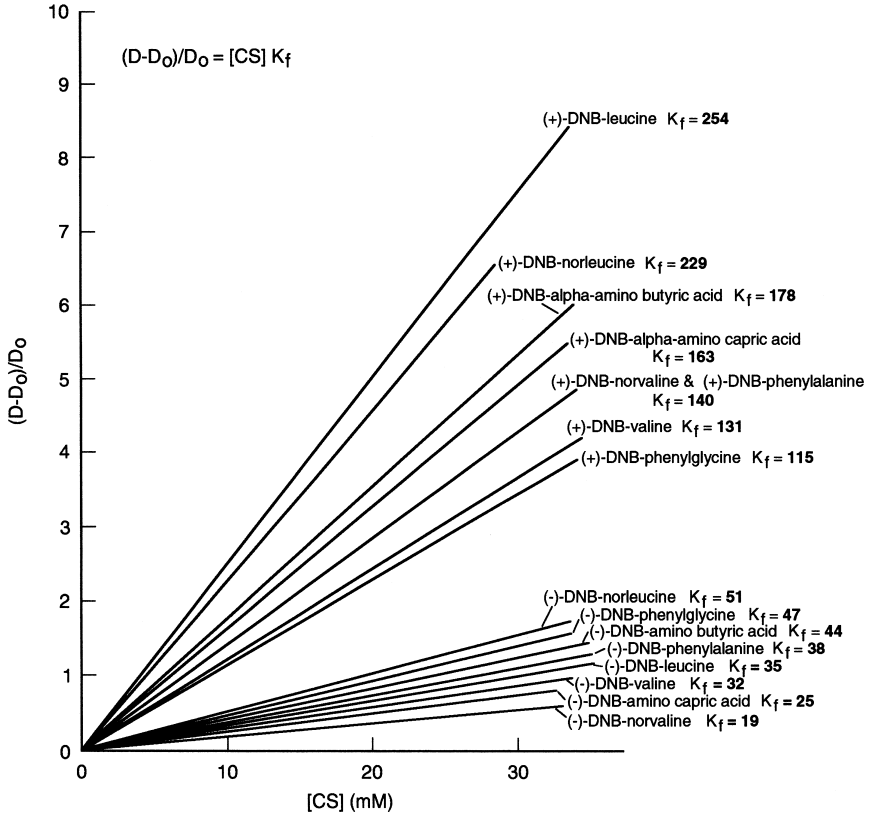

Figure 2. Formation constants (K_f) of CS-DNB amino acids (CS: N-dodecanoyl-L-proline-3,5-dimethylanilide). K_f values change with the length of hydrocarbon chains at the site of the asymmetric carbon of the DNB amino acids. The largest K_f value is obtained from (+)-DNB-leucine.

Combining Eqs. 6 and 9, α can be expressed as:

$$\alpha = (1 + K_{F+}[CS]_{org})/1 + K_{F-}[CS]_{org}) \quad (10)$$

The experimental distribution factor, $D_{\pm}$, is related to the whole solute-stationary phase interactions, polar and chiral. The enantioselectivity factor is

Table 2

Effect of the CS Substitution on DNB-Leucine Chiral Recognition

N-Dodecanoyl-L-proline	V_- (mL)	V_+ (mL)	Kf^- (L mol^{-1})	Kf^+ (L mol^{-1})	α	$\Delta(\Delta G_c)$ (kJ mol^{-1})
4-Methylanilide	208	467	26	237	3.8	3.30
3,5-Dimethylanilide	219	488	35	254	3.6	3.16
3-Methylanilide	229	418	43	197	2.7	2.42
Anilide	236	373	49	160	2.1	1.86
2,4-Dimethylanilide	233	262	46	70	1.25	0.55
2-Methylanilide	207	222	25	37	1.16	0.37

DNB: 3,5-dinitrobenzoyl; $V_\pm$: retention volume of the corresponding enantiomer at [CS] = 0.02 M; $K_{f\pm}$: formation constant of the CS-enantiomer complex; α: enantioselectivity ratio at [CS] = 0.02 M; $\Delta(\Delta G_c)$: difference in the molecular free energy of the chiral interaction between the + (L) and - (D) enantiomers with 0.02 M (8 g/L) CS concentration. The data correspond to the Figure 3 chromatograms.

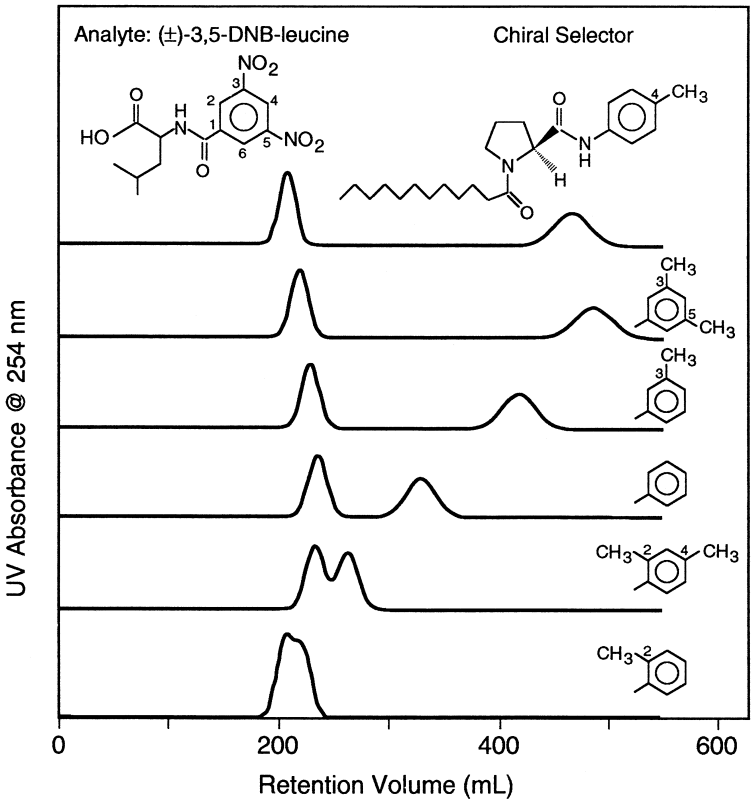

Figure 3. Effect of the position and/or number of methyl group(s) on the CS benzene ring. CCC chromatograms obtained after injection of (±)-DNB-leucine. Mobile phase: lower aqueous phase of the system composed of hexane/ethyl acetate/methanol/water (8:2:5:5, v/v/v/v) acidified with HCl at 0.1M, pumped at 3 mL/min from head to tail. Stationary phase: upper organic phase of the above biphasic system containing different chiral selectors (N-dodecanoyl-L-proline-dimethyl- and monomethyl-anilides) each at 0.02 M concentration (ca 8 g/L) as indicated in the right part of the figure. Centrifuge rotation speed: 800 rpm; column capacity V_c: 315 mL, stationary phase volume V_s: 215 mL, mobile phase volume V_m: 115 mL. See Table 2.

related to the difference in molecular free energy of the chiral interaction for the two enantiomers.[9,10] This difference, $\Delta(\Delta\_G_c)$, can be calculated by:[9]

$$\Delta(\Delta G_c) = 2RT \ln \alpha \qquad (11)$$

The $\Delta(\Delta G_c)$ energy is an accurate measure of the degree of chiral recognition between two enantiomers and a given CS. From a mechanistic point of view, the

$\Delta(\Delta G_c)$ energy values listed in Table 1 suggest that a methylene group, $-CH_2-$, directly connected to the asymmetric carbon favors the interaction with the CS molecule.

The data obtained with the Figure 2 experiments further suggest that the interaction is further enhanced when a linear three methylene groups is attached to the asymmetric carbon with the optimal interaction with the $-CH_2-CH(CH_3)-CH_3$ leucine alkyl group.

In the chiral recognition mechanism just studied, changing the amino acid was acting on the interaction between the alkyl side chain of the amino acid and the blocked alkyl ring in the pyrrolidine cycle of the proline derivatized CS molecule. The capability of the CCC method is shown in the study of the effect of structural variations in the π-basic site of the CS molecule.

The position and/or the number of methyl groups on the benzene ring of the CS molecule were changed as shown by Figure 3. The CCC chromatograms obtained with (±)-3,5-DNB-leucine clearly show that the interaction between the π-basic group in the chiral selector and the dinitro π-acid group in the enantiomer plays an important role in governing the enantioselectivity.

Table 2 lists the $K_{f\pm}$ formation constants, the α factor and the $\Delta(\Delta G_c)$ chiral energies for the six different CS molecules prepared.

Large K_{f+} and $\Delta(\Delta G_c)$ values are obtained either by exactly matching the positions of the $-NO_2$ and $-CH_3$ groups at positions 3 and 5 or by placing a single $-CH_3$ at position 4 between the two $-NO_2$ groups in positions 3 and 5.

On the other hand, placing the $-CH_3$ at position 2 on the benzene ring adversely affects the K_f and $\Delta(\Delta G_c)$ values, probably by interfering with the π–π electron interaction between the two benzene rings.

The CCC apparatus is available from several companies (Pharma-Tech Research Corporation, Baltimore, USA; P.C. Inc., Potomac, MD, USA; Conway Centri Chrom, Buffalo, NY, USA; Shimadzu Corporation, Kyoto, Japan) and works well at the low levels of sample as shown above.

Therefore, the simple and reliable method of K_f measurement for enantiomer-chiral selector complexes presented above should be useful for investigating the mechanisms of enantioselectivity, which should lead to design of more efficient chiral selectors.

ACKNOWLEDGMENT

The authors are indebted to Dr. Henry M. Fales for editing the manuscript.

REFERENCES

1. C. J. Welch, J. Chromatogr. A, **666**, 3-26 (1994).

2. A. Shibukawa, D. K. Lloyd, I. W. Wainer, Chromatographia, **35**, 419-429 (1993).

3. L. Oliveros, P. Franco Puertolas, C. Minguillon, E. Camacho-Frias, A. Foucault, F. Le Goffic, J. Liq. Chromatogr., **17**, 2301 (1994).

4. Y. Ito, R. L. Bowman, Science, **167**, 81-283 (1970).

5. Y. Ito, in **Countercurrent Chromatography: Theory and Practice**, N. B. Mandava, Y. Ito, eds., Marcel Dekker, New York, 1988, Ch. 3, pp. 79-442.

6. W. D. Conway, **Countercurrent Chromatography: Apparatus, Theory and Applications**, VCH, New York, 1989.

7. Y. Ma, Y. Ito, A. Foucault, J. Chromatogr. A, **704**, 75-81 (1995).

8. Y. Ma, Y. Ito, Anal. Chem., **67**, 3069-3074 (1995).

9. A. Berthod, C. D. Chang, D. W. Armstrong, Anal. Chem., **64**, 873-879 (1992).

10. A. Berthod, C. D. Chang, D. W. Armstrong, Anal. Chem., **64**, 395-404 (1992).

Received April 27, 1999
Accepted May 16, 1999
Manuscript 5066

Request Permission or Order Reprints Instantly!