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Chromatographic behaviour of cyclodextrin complexes of NADH and NADP

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SUMMARY

The retention behaviour of NADH and NADP on an ODS-C_{18} reversed-phase column was investigated by using a 0.05 *M* phosphate buffer (pH 6) containing α-, β- or γ-cyclodextrin (CD) as the mobile phase. Three reversible processes were assumed and the partition coefficients, k_2, of inclusion complexes and their stability constants, *K*, were calculated. The value of k_2 is clearly dependent on the molecular weight of the CD. It was established from the calculated values of *K* that NADH and NADP are liable to form more stable inclusion complexes with β-CD than α- and γ-CDs. NADP has a higher stability constant than NADH owing to the formation of hydrogen bonds between the phosphate moieties of NADP and the hydroxyl groups of CDs.

INTRODUCTION

α-, β- and γ-cyclodextrins (CDs) are cyclic oligosaccharides containing six, seven and eight glucose units, respectively. Because of the dimensions of their non-polar central cavities, CDs act as "hosts", forming stable inclusion complexes with a variety of "guest" species. The formation of an inclusion complex is based on the ability of the CD to incorporate hydrophobic molecules in its cavity and to form hydrogen bonds between hydroxyl groups at the entrance of the CD cavity and the hydrophilic moieties of the guest molecule[1–3]. For example, a chiral molecule forms a hydrogen bond with the 2-hydroxyl groups at the entrance of the CD cavity[4,5]. In addition, many other factors such as Van der Waals force, dipole–dipole interactions and hydrophobic interactions also play important roles in governing the stability of the complex[6].

In recent years, CD inclusion phenomena have been utilized in high performance liquid chromatographic (HPLC) techniques in two ways: the use of CD-bonded stationary phases as reported by Issaq[7] and the use of CD as one component of the mobile phase in reversed-phase liquid chromatography (RP-LC)[8–11]. Cline Love and Arunyanart[12] dealt with the latter method and calculated the stability constants, *K*, of benzene, phenol, *o*, *m*- and *p*-nitrophenol, naphthalene and biphenyl with β-CD.

This paper reports the results of further studies on the determinations of the

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capacity factors, k'_2, of inclusion complexes formed between NADH or NADP and CDs, and their stability constants, K, based on a model consisting of three reversible processes[13,14]. The elution behaviour of NADH and NADP in RP-LC was also investigated by using a 0.05 *M* phosphate buffer (pH 6) containing α-, β- or γ-CD as the mobile phase.

EXPERIMENTAL

Reagents

NADP (nicotinamide adenine dinucleotide phosphate) and NADH (nicotinamide adenine dinucleotide) were obtained from Sigma and α-, β- and γ-CDs from Tokyo Kasei. All the materials were used without further purification.

Apparatus and procedures

A Bionert LC system (Japan Spectroscopic) equipped with a Model 875-UV Intelligent UV–VIS detector operating at 254 nm, an SIC Chromatocorder 12 and a Model 880-PU Intelligent pump were employed. A Hitachi Gel 3056 ODS-C_{18} reversed-phase column (150 × 4 mm I.D.; 5 μm particle diameter) was used.

The CD mobile phase was prepared by dissolving the appropriate weight of CD in 0.05 *M* phosphate buffer (pH 6) (Na_2HPO_4–NaH_2PO_4) and filtering through a poly(vinylidene fluoride) membrane filter (Millipore, pore size 0.65 μm). All the chromatographic experiments were carried out at a constant flow-rate of 1.0 ml/min and at room temperature. A 3-μl sample volume containing 50 μg/ml of solute was injected into the column.

The void volume of the column with a mobile phase containing different concentrations of the CDs was determined by using analytical-reagent grade potassium nitrate[13].

RESULTS AND DISCUSSION

The elution behaviour of NADH and NADP can be analysed by using a model consisting of three reversible reactions: a reversible reaction of guest molecule A and CD in the mobile phase to form an inclusion complex A · CD, and two reversible adsorption processes of A and A · CD on the stationary phase of the ODS-C_{18} column. A and A · CD adsorbed on the ODS-C_{18} column are represented by A_s and A · CD_s, respectively.

$$A + CD \overset{K}{\rightleftharpoons} A \cdot CD \tag{1}$$

$$A \overset{k_1}{\rightleftharpoons} A_s \tag{2}$$

$$A \cdot CD \overset{k_2}{\rightleftharpoons} A \cdot CD_s \tag{3}$$

where K is the stability constant of the inclusion complex A · CD and k_1 and k_2 are the partition coefficients of A and A · CD, respectively, between the mobile phase and the stationary phase.

The capacity factors of A and A · CD, k'_1 and k'_2, are given as follows[15]:

$$k'_1 = \varphi k_1 = \varphi \frac{[A_s]}{[A]} \tag{4}$$

$$k'_2 = \varphi k_2 = \varphi \frac{[A \cdot CD_s]}{[A \cdot CD]} \tag{5}$$

where φ is the phase ratio, *i.e.*, the ratio of the volume of the stationary phase, V_s, to the void volume of the mobile phase, V_0, in the column.

The capacity factor k' of the guest molecule A, which is experimentally determined, is represented by[15]

$$k' = \varphi \frac{[A_s] + [ACD_s]}{[A] + [ACD]} \tag{6}$$

Combination of eqns. 1, 4, 5 and 6 yields the following expression for the capacity factor:

$$k' = \frac{k'_1 + k'_2 K[CD]}{1 + K[CD]} \tag{7}$$

where [CD] is the molar concentration of CD. Eqn. 7 is similar to that derived by Uekama *et al.*[16] for the determination of the stability constants of the CD complexes of various ionic species measured by ion-exchange chromatography.

If k'_2 is small enough to be ignored, the following expression is obtained:

$$k' = \frac{k'_1}{1 + K[CD]} \tag{8}$$

By taking the reciprocal of both sides of eqn. 8 and plotting $1/k'$ *vs.* [CD], one can obtain a straight line and the value of the stability constant K is obtained as the slope/intercept ratio.

If k'_2 cannot be neglected, eqn. 7 can be linearized by a simple transformation:

$$\frac{k'_1 - k'_2}{k' - k'_2} = 1 + K[CD] \tag{9}$$

When one takes an appropriate value of k'_2 in eqn. 9, the plot of $(k'_1 - k'_2)/(k' - k'_2)$ *vs.* [CD] should be linear and the value of the stability constant K is obtained from the slope of the line.

The retention volumes of NADP and NADH were measured at different concentrations of α-, β- or γ-CD in the 0.05 M phosphate buffer (pH 6). The capacity factor k' for each guest molecule was calculated from the retention data by using the ratio $(V_R - V_0)/V_0$, where V_R is the elution volume of the guest molecule and V_0 is the void volume of the column. The relationships between the capacity factor k' of NADP

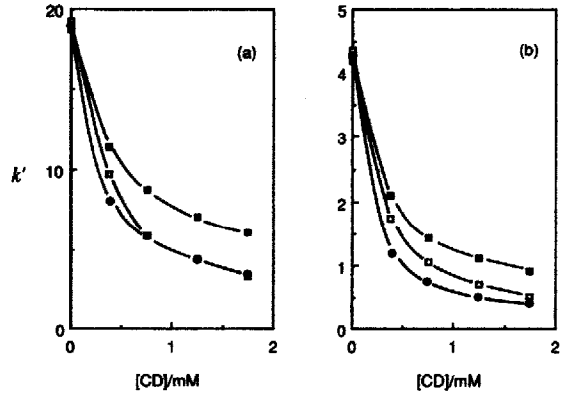

Fig. 1. Relationships between the experimental capacity factors k' of (a) NADH and (b) NADP to the concentrations of (□) α-CD, (●) β-CD and (■) γ-CD in 0.05 *M* phosphate buffer (pH 6) mobile phase.

and NADH and the concentration of α-, β- or γ-CD in the mobile phase are shown in Fig. 1. With increasing concentration of α-, β- or γ-CD, the k' values of NADH and NADP are reduced. It is evident that α-, β- and γ-CD form inclusion complexes with NADH and NADP. Addition of β-CD reduces the k' value more than addition of α- or γ-CD, and the effect decreases in the order β-CD > α-CD > γ-CD. On the other hand, the k' values of NADP are reduced more than those of NADH, NADP is considered to form more stable complexes than NADH with CDs.

Fig. 2 shows the relationships between the reciprocal capacity factors of (a) NADH and (b) NADP and the concentration of α-, β- or γ-CD in the 0.05 *M* phosphate buffer (pH 6) mobile phase. The $1/k'$ of NADH and NADP *vs.* [α-CD] plots satisfy a linear relationship. This shows that the value of k'_2 is adequately small for α-CD. The stability constant K for α-CD was calculated from this plot as shown in Table I. For β- and γ-CD, the plots of $1/k'$ *vs.* [CD] were not linear, indicating that the capacity factor k'_2 of β- and γ-CD cannot be neglected. Therefore, values of k'_2 were

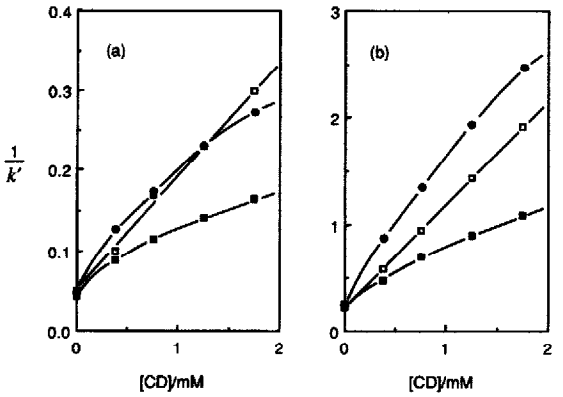

Fig. 2. Relationships between the reciprocal capacity factor of (a) NADH and (b) NADP and the concentration of (□) α-CD, (●) β-CD and (■) γ-CD in the 0.05 *M* phosphate buffer (pH 6) mobile phase. In the case of α-CD, the solid straight lines were obtained.

TABLE I

PARTITION COEFFICIENTS k_1 AND k_2 AND STABILITY CONSTANTS K (mmol l^{-1}) OF NADH AND NADP WITH α-, β- AND γ-CD

Compound	*α-CD*			*β-CD*			*γ-CD*		
	k_1	k_2	K	k_1	k_2	K	k_1	k_2	K
NADH	34.86	0.0	2.80	34.86	2.96	4.42	34.86	5.48	2.37
NADP	7.73	0.0	4.36	7.73	0.18	7.15	7.73	0.58	3.10

assigned to give a linear relationship between $(k'_1 - k'_2)/(k' - k'_2)$ and the concentration of β- or γ-CD in the mobile phase according to eqn. 9. The resulting relationships are shown in Fig. 3. The stability constants K for β-CD and γ-CD were determined from the slopes.

The results are summarized in Table I. The k'_1 and k'_2 values are converted to the k_1 and k_2 values, respectively, by using eqns. 4 and 5. The stability constants K of β-CD with NADP and NADH are large compared with those of α- and γ-CD. This means that NADP and NADH are liable to form stable inclusion complexes with β-CD. Moreover, the K value of NADP is always larger than that of NADH.

The formation of the inclusion complexes is due to the ability of CDs to incorporate hydrophobic molecules in their cavities and to form hydrogen bonds between the hydroxyl groups at the entrance of the CD cavity and the polar groups of the guest molecule[1–5]. It is considered that NADH and NADP form inclusion complexes with CD by incorporating their adenine moiety in the CD cavity, as shown schematically in Fig. 4. The cavity size of β-CD matches well the size of the adenine moiety, because the inner diameters of α-, β- and γ-CD are 0.57, 0.78 and 0.95 nm, respectively[17], and the lateral maximum size of the adenine moiety of NADH and NADP was estimated from the bond length and bond angles of the adenine molecule to be *ca.* 0.62 nm.

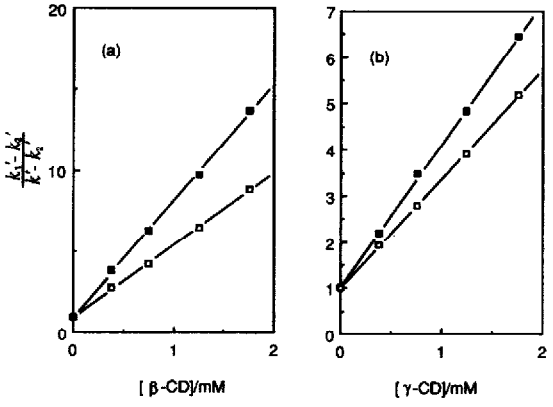

Fig. 3. Relationships between $(k'_1 - k'_2)/(k' - k'_2)$ and the concentrations of (a) β-CD and (b) γ-CD in the mobile phase for (□) NADH and (■) NADP. Solid lines were obtained by plotting eqn. 9.

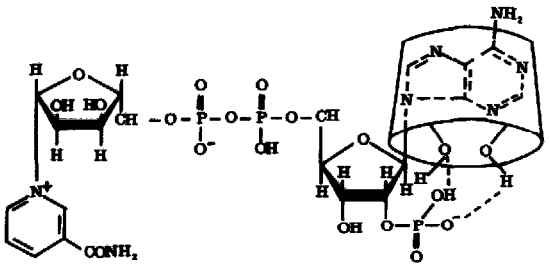

Fig. 4. Schematic representation of the NADP–CD inclusion complex. The dashed lines represent the hydrogen bonds.

The partition coefficients k_2 of γ-CD complexes are larger than those of β-CD complexes, and the partition coefficients k_2 of α-CD complexes are nearly zero in this instance. The value of k_2 is clearly dependent on the molecular weight of the CD and an increase in the molecular weight enhances the hydrophobicity and therefore the column affinity of CD complexes. Lastly, it can be noted that NADP forms hydrogen bonds between its phosphate moiety and the hydroxyl groups of CD, as shown in Fig. 4, resulting in a larger stability constant K than for NADH.

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