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# Note

## Chromatographic behaviour of cyclodextrin complexes of nucleotides, nucleosides and their bases

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The α-, β- and γ-cyclodextrins (CDs) are cyclic oligosaccharides containing six, seven and eight glucose units, respectively. The specific ability of CDs to form inclusion complexes with a variety of molecules and ions has been utilized in high-performance liquid chromatography (HPLC) for the separation of optical, geometrical and structural isomers [1–3]. In recent years, preparations of CD-bonded stationary phases [1–8] and applications of mobile phases containing CDs in reversed-phase liquid chromatography (RP-LC) [9–11] have been widely studied. Cline Love and Arunyanart [12] dealt with the latter method to determine the stability constants ($K$) of the inclusion complexes of benzene, phenol, *o*, *m*- and *p*-nitrophenol, naphthalene and biphenyl with β-CD according to a three-phase model of micellar chromatography [13,14]. Moreover, the separations of structural isomers of hydroxy-, methoxy- and amino-substituted benzoic acids using a mobile phase containing β-CD or a β-CD-immobilized stationary phase were reported by Bazánt *et al.* [15].

We have recently used α-, β- and γ-CDs as mobile phase components and studied the separation of NADH and NADP using a reversed-phase chromatographic system. The capacity factors ($k'_2$) of inclusion complexes of NADH and NADP with CDs, and their stability constants were determined based on a model consisting of three reversible processes [16].

This paper reports the results of further studies on the determination of the capacity factors of inclusion complexes of nucleotides (AMP, ADP and ATP), nucleosides (adenosine, uridine and thymidine) and bases (adenine, uracil and thymine) with CDs, and their stability constants by chromatography [16–20]. This method is useful for the analysis, separation and purification of these nucleotides, nucleosides and bases.

## EXPERIMENTAL

### *Reagents*

AMP (adenosine 5′-monophosphate), ADP (adenosine 5′-diphosphate) and ATP (adenosine 5′-triphosphate) were obtained from Sigma, adenosine, uridine,

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adenine, uracil and thymine from Wako and thymidine and $\alpha$-, $\beta$- and $\gamma$-CDs from Tokyo Kasei. All 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 reversed-phase column (150 × 4 mm I.D.; 5 $\mu$m particle diameter) was used.

The CD mobile phase was prepared by dissolving an appropriate weight of CD in 0.05 *M* $Na_2HPO_4$–$NaH_2PO_4$ buffer (pH 6) and filtering through a poly(vinylidene fluoride) membrane filter (Millipore, pore size 0.65 $\mu$m). All the chromatographic experiments were carried out at a constant flow-rate of 1.0 ml/min and at room temperature. A 3-$\mu$l sample solution containing 50 $\mu$g/ml of solute was injected into the column. The void volume of the column with a mobile phase containing different concentrations of CD was determined by using analytical-reagent grade potassium nitrate [17].

## RESULTS AND DISCUSSION

An example of separations of nucleotides (ADP and ATP), nucleosides (uridine and thymidine) and bases (uracil and thymine) using 0.05 *M* phosphate buffer (pH 6) solution with or without $\beta$-CD is shown in Fig. 1. By adding $\beta$-CD to the mobile phase the retention times of these solutes were considerably reduced, and the peaks of ADP

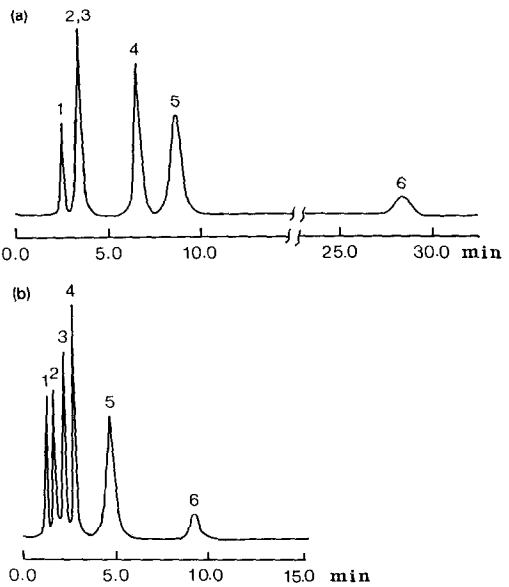


Fig. 1. Separation of nucleotides, nucleosides and bases by using a mobile phase of 0.05 *M* phosphate buffer (pH 6), (a) with and (b) without CD. Column, Hitachi Gel 3056 ODS-$C_{18}$; flow-rate, 1.0 ml/min. Peaks: 1 = ATP; 2 = ADP; 3 = uracil; 4 = uridine; 5 = thymine; 6 = thymidine.

and uracil were separated from one another. Similar behaviour was observed on addition of α- and γ-CD to the phosphate buffer mobile phase.

The stability constant and capacity factor of a CD inclusion complex can be calculated by using eqn. 1, which was derived earlier [16] on the basis of a three reversible reaction model consisting a reversible complex formation process between the guest molecule and CD in the mobile phase, and two reversible adsorption processes between the guest molecule and the inclusion complex on the stationary phase of an ODS column:

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

where $k'$ is the experimentally determined capacity factor, $K$ is the stability constant of the inclusion complex and $k'_1$ and $k'_2$ are the capacity factors of the free guest molecule and its inclusion complex, respectively. If $k'_2$ is small enough to be ignored, the value of $K$ is obtained from straight lines obtained by plotting $1/k'$ *vs.* [CD]. If $k'_2$ cannot be neglected, eqn. 1 is rearranged to

$$\frac{k'_1 - k'_2}{k' - k'_2} = 1 + K[\mathrm{CD}] \tag{2}$$

and by assigning an appropriate value of $k'_2$ to obtain a straight line of $(k'_1 - k'_2)/(k' - k'_2)$ *vs.* [CD], the value of $K$ is determined from the slope of the line.

The retention volumes of nucleotides (AMP, ADP and ATP), nucleosides (adenosine, uridine and thymidine) and their bases (adenine, uracil and thymine) were measured at different concentrations of α-, β- and γ-CD in 0.05 *M* phosphate buffer (pH 6). The relationships between the capacity factors of nucleotides, nucleosides and bases and the concentration of α-, β- or γ-CD in the mobile phase are shown in Figs. 2, 3 and 4, respectively. Similar relationships were observed previously for NADH and NADP [16]. With an increasing concentration of α-, β- or γ-CD, the $k'$ values of nucleotides, nucleosides and their bases are reduced, suggesting that α-, β- and γ-CDs

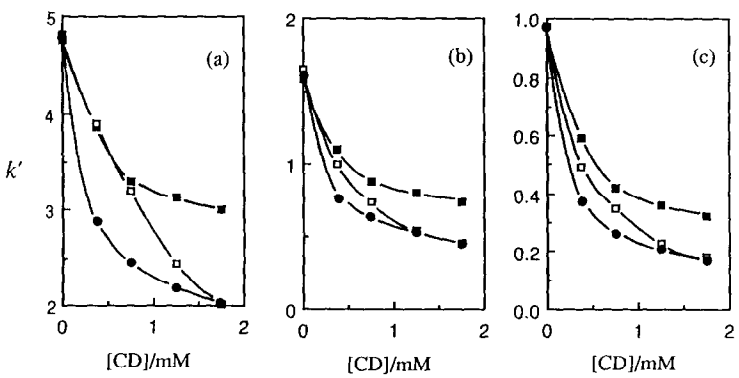


Fig. 2. Relationships between the capacity factors, $k'$, of (a) AMP, (b) ADP and (c) ATP and the concentrations of (□) α-CD, (●) β-CD and (■) γ-CD. Conditions as in Fig. 1.

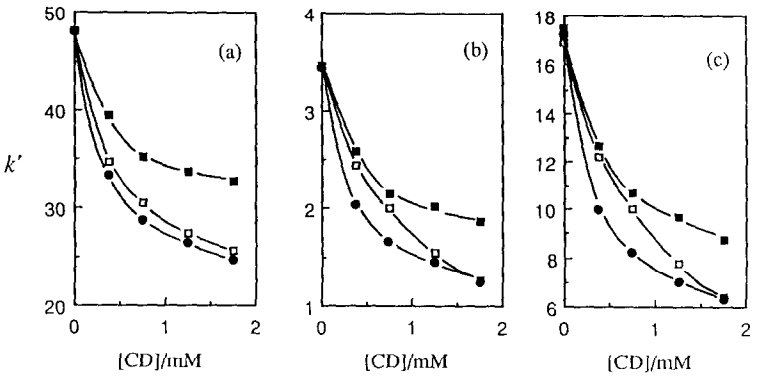


Fig. 3. Relationships between the capacity factors, $k'$, of (a) adenosine, (b) uridine and (c) thymidine and the concentrations of (□) α-CD, (●) β-CD and (■) γ-CD. Conditions as in Fig. 1.

form inclusion complexes with these compounds. Addition of β-CD reduces the $k'$ values more than additions of α- and γ-CDs, and the effects decrease in the order β-CD > α-CD > γ-CD.

Plots of $1/k'$ *vs.* [α-CD] for AMP, ADP, ATP, uridine, thymidine, uracil and thymine give good linear relationships. In these instances the values of $k'_2$ are adequately small. The stability constants of these compounds with α-CD were calculated from these compounds with α-CD were calculated from these plots and the results are given in Table I. The partition coefficients $k_1$ and $k_2$ were determined from the capacity factors $k'_1$ and $k'_2$, respectively [16], and these values are also given in Table 1. The order of the partition coefficients $k_1$ is adenine > adenosine ≫ AMP > ADP > ATP in the purine series and thymidine > thymine > uridine > uracil in the pyrimidine series. On the other hand, the order is adenosine > thymidine > uridine in the nucleoside series and adenine > thymine > uracil in the base series.

ATP was eluted more rapidly than ADP and both were eluted faster than AMP. This is because of the larger number of phosphate groups in the compounds, which

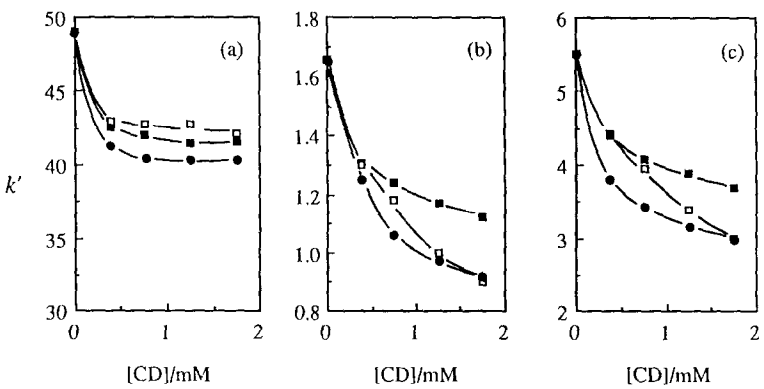


Fig. 4. Relationships between the capacity factors, $k'$, of (a) adenine, (b) uracil and (c) thymine and the concentrations of (□) α-CD, (●) β-CD and (■) γ-CD. Conditions as in Fig. 1.

TABLE I

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

| Compound | α-CD | | | β-CD | | | γ-CD | | |
|---|---|---|---|---|---|---|---|---|---|
| | $k_1$ | $k_2$ | $K$ | $k_1$ | $k_2$ | $K$ | $k_1$ | $k_2$ | $K$ |
| *Nucleotides* | | | | | | | | | |
| AMP | 8.51 | 0.0 | 0.93 | 8.51 | 2.96 | 3.13 | 8.51 | 4.66 | 2.27 |
| ADP | 3.00 | 0.0 | 1.54 | 3.00 | 0.53 | 3.76 | 3.00 | 1.00 | 2.52 |
| ATP | 1.77 | 0.0 | 2.51 | 1.77 | 0.11 | 4.52 | 1.77 | 0.35 | 2.80 |
| *Nucleosides* | | | | | | | | | |
| Adenosine | 87.70 | 34.72 | 1.67 | 87.70 | 37.00 | 2.94 | 87.70 | 53.35 | 2.49 |
| Uridine | 6.32 | 0.0 | 0.97 | 6.32 | 1.75 | 3.57 | 6.32 | 2.70 | 2.29 |
| Thymidine | 31.28 | 0.0 | 0.93 | 31.28 | 8.13 | 3.08 | 31.28 | 10.96 | 1.74 |
| *Bases* | | | | | | | | | |
| Adenine | 89.62 | —[a] | —[a] | 89.62 | —[a] | —[a] | 89.62 | —[a] | —[a] |
| Uracil | 3.03 | 0.0 | 0.44 | 3.03 | 1.39 | 2.68 | 3.03 | 1.86 | 2.67 |
| Thymine | 10.05 | 0.0 | 0.45 | 10.05 | 4.75 | 3.50 | 10.05 | 5.85 | 1.98 |

[a] The peak of adenine was so broad and diffuse that $k'$ was difficult to determine.

influences their hydrophilicity and solubility. The retention order of AMP, ADP, and ATP is analogous to that obtained on a μBondapak $C_{18}$ reversed-phase column by using 0.05 *M* ammonium dihydrogenphosphate buffer as the mobile phase at pH 6.0 and a flow-rate of 2 ml/min [21]. In the pyrimidine series, the nucleosides were retained more strongly than their bases on the ODS columns; even so, the nucleosides have a higher hydrophilicity than their bases owing to the hydroxyl group on the ribose ring [21–23]. In reversed-phase chromatography, the elution characteristics are usually dependent on the hydrophobic interaction between the hydrophobic surface of the stationary phase and the substrate molecules in the aqueous phase and, therefore, the retention order observed for the nucleosides and the bases can be explained by this theory [24–26]. Adenosine and adenine have substantial hydrophobic interactions between their purine ring and the stationary phase of column compared with the pyrimidine ring series, as the purine ring is more hydrophobic than the pyrimidine ring. In the same manner, thymidine and thymine are more hydrophobic and retained more strongly than uridine and uracil because of the methyl group on their ring.

With adenosine, the plot of $1/k'$ *vs.* [CD] was not linear, indicating that $k'_2$ is not negligibly small. The $k'_2$ values of the inclusion complexes of the nucleotides, the nucleosides and their bases with β- and γ-CD also cannot be neglected. The values of $k'_2$ were therefore assigned to give a linear relationship between $(k'_1 - k'_2)/(k' - k'_2)$ and the concentration of [CD] in the mobile phase according to eqn. 2. The stability constants of all the nucleotides, nucleosides and bases with β- and γ-CDs together with that of adenosine with α-CD were determined from the slopes of these plots and the results are also given in Table I. The elution peak of adenine was very broad and diffuse, probably because it is slightly soluble in aqueous solution. Hence it was difficult to determine its stability constant.

The stability constants of these nucleotides, nucleosides and bases with β-CD are larger than those with α- and γ-CDs. This indicates that these nucleotides, nucleosides

and bases are liable to form more stable inclusion complexes with β-CD. The relative stabilities of CD inclusion complexes are governed by factors such as hydrogen bonding, hydrophobic interactions and solvation effects and also the space-filling ability of the guest molecules [27–31]. It was confirmed that a better fit of the substrate molecule in the cavity results in stronger complex formation [32]. The cavity sizes of α-, β- and γ-CDs are reported to be 0.45–0.57, 0.70–0.78 and 0.85–0.95 nm [33–35], respectively, and the sizes of adenine, thymine and uracil are estimated to be about 0.70, 0.66 and 0.58 nm, respectively, from the CPK model. The experimental results and the calculated data confirm that these nucleotides, nucleosides and bases form inclusion complexes with β- and γ-CDs by incorporating their purine or pyrimidine ring into the cavity of the respective CD. On the other hand, the inner diameter of the cavity of α-CD is to small to incorporate the purine and pyrimidine rings completely. Probably these rings are partly incorporated into the cavity of α-CD. This explains the small $K$ values of inclusion complexes with α-CD shown in Table I.

The adsorption of the inclusion complexes of these nucleotides, nucleosides and bases on the ODS stationary phase is weak compared to that of the corresponding free solutes, that is, $k_1 > k_2$. The partition coefficients $k_2$ of γ-CD complexes are larger than those of β-CD complexes, and those of α-CD complexes are nearly zero except for adenosine. Adenosine is more hydrophobic than the other nucleosides, as suggested by its large value of $k_1$ [26], so that it is assumed that its α-CD inclusion complex is strongly adsorbed on the column and the $k_2$ value of adenosine is far from zero. The value of $k_2$ is clearly dependent on the molecular weight or the cavity size of CD and an increase in the molecular weight enhances the affinity of CD complexes to the ODS column.

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