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## Note

# Indirect photometric detection of cyclodextrins via inclusion complexation in micro high-performance liquid chromatography

TOYOHIDE TAKEUCHI, MASAHIRO MURAYAMA and DAIDO ISHII*
*Department of Applied Chemistry, Faculty of Engineering, Nagoya University, Chikusa-ku, Nagoya 464 (Japan)*

The unique features of cyclodextrins in various pharmaceutical areas have been widely acknowledged in recent years. An improvement in the stability or solubility of drugs using cyclodextrins has been reported, which is due to the inclusion complexation properties of cyclodextrins. For example, cyclodextrins form inclusion complexes with barbiturates, which improves their solubility and results in increase in gastrointest inal absorption[1–7]. A soluble powder for injections of prostaglandin-El stabilized with α-cyclodextrin is commercially available.

Although many papers have reported the parenteral application of cyclodextrins[9,10], few have discussed the bioanalysis of cyclodextrins[11,12]. The development of methods for the microdetermination of cyclodextrins in plasma is necessary for pharmacokinetic studies. Cyclodextrins show almost no UV absorption. Only refractive index detection can be used, but this method is generally not sensitive. Indirect detection is a good alternative.

Various detection principles have been reported for the visualization of transparent analytes in high-performance liquid chromatography (HPLC), among which indirect photometric detection has most successfully been applied in ion chromatography[13]. In indirect detection, analytes are visualized via interaction with the mobile phase component or postcolumn interaction. In the former case the analyte is visualized by variation of a background due to the mobile phase component, while in the latter case the mobile phase component is transparent to the detector and the secondary species produced by the postcolumn interaction corresponding to the analytes are detected. Indirect detection usually refers to the former case, and postcolumn ion replacement[14–16] and postcolumn enzyme reaction can be included in the latter case[17].

Frijlink *et al.*[12] reported a new method for indirect photometric detection of cyclodextrins via postcolumn complexation with phenolphthalein. The detection principle was based on the finding that the colour intensity of phenolphthalein decreased on formation of the inclusion complex with cyclodextrins[18–20]. The detection limit for β-cyclodextrin was 1.0 μg/ml (ref. 12) and it was suggested that this would be improved by decreasing the noise level due to incomplete postcolumn mixing.

An advantage of micro HPLC lies in the ability to improve the mass detection limit. This is especially important when the amount of sample available is limited. This paper will describe the indirect detection of cyclodextrins via inclusion complexation

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with phenolphthalein in micro HPLC, and the improvement of the mass detection limits of cyclodextrins will be demonstrated.

## EXPERIMENTAL

### *Apparatus*

The liquid chromatograph comprised an MF-2 Micro Feeder pump (Azumadenki Kokyo, Tokyo, Japan) equipped with an MS-GAN 050 gas-tight syringe (0.5 ml; Ito, Fuji, Japan), an ML-425 micro valve injector (20 nl; JASCO, Tokyo, Japan), a micropacked separation column dipped in a laboratory-made water-bath, an UVIDEC-100 III spectrophotometer (JASCO) and a RC-128 chart recorder (JASCO).

The separation column comprised fused-silica tubing (100 mm × 0.35 mm I.D.) packed with commercially available alkyl-modified silica, Develosil ODS-3K (3 μm; Nomura Chemical, Seto, Japan) or Capcell Pak $C_{18}$ (5 μm; Shiseido, Tokyo, Japan). The latter material is resistant to alkaline solutions up to pH 10. The detector was set at 550 nm and operated with an offset of 0.12–0.18 a.u.

### *Reagents*

α- and β-cyclodextrin were obtained from Tokyo Chemical Industry (Tokyo, Japan), γ-cyclodextrin from Wako Pure Chemical Industries (Osaka, Japan). Other reagents were supplied by Wako Pure Chemical Industries. All the reagents except for HPLC-grade distilled water were of reagent grade, and were employed without any treatment.

## RESULTS AND DISCUSSION

A chromatogram of β- and γ-cyclodextrin is shown in Fig. 1. The Develosil ODS-3K column gave an higher efficiency than the Capcell Pak $C_{18}$ column. The


Fig. 1. Indirect photometric detection of cyclodextrins. Column: Develosil ODS-3K, 100 mm × 0.35 mm I.D. Mobile phase: 0.3 m*M* phenolphthalein dissolved in 3% methanol solution, pH 12.2. Flow-rate: 2.8 μl/min. Samples: β = β-cyclodextrin; γ = γ-cyclodextrin; 2.0 m*M* of each was injected. Sample volume: 20 nl. Wavelength of detection: 550 nm.

sample concentration was 2.0 m*M*, corresponding to an injected amount of 40 pmol each. The peak area of $\beta$-cyclodextrin was 1.6 times larger than that of $\gamma$-cyclodextrin, and the value was smaller than that observed by the flow injection method, *viz.*, 3.9. If the cyclodextrins are visualized only by inclusion complexation, the peak area ratio should coincide with that observed by the flow injection method. This inconsistency may be explained by the fact that the cyclodextrins perturb the partitioning of phenolphthalein during the chromatographic process, which also contributed to the visualization of the cyclodextrins based on the common indirect detection principle of uncharged species[21,22]. The positive peak, denoted as "S" in Fig. 1, was eluted in 13 min, and may be produced by perturbation of the partitioning of phenolphthalein due to the cyclodextrins.

In addition, $\alpha$-cyclodextrin was not detected under the operating conditions in Fig. 1. The background of the mobile phase was kept at around 0.18 a.u. by considering the linearity of the signal[23].

The concentration of methanol in the mobile phase affected the mass detection limit, because it can influence the stability of the inclusion complex and the retention times of the analytes as well as of phenolphthalein. A concentration of 3% was selected because the system peak and the cyclodextrins were separated in a reasonable time.

The mass detection limits under the operating conditions in Fig. 1 were 0.58 pmol (or 0.66 ng) for $\beta$-cyclodextrin and 0.86 pmol (or 1.1 ng) for $\gamma$-cyclodextrin at a signal-to-noise ratio of 2. These values represent a marked improvement compared with those achieved by Frijlink *et al.*[12], *e.g.*, 1.0 $\mu$g for $\beta$-cyclodextrin. This is due mainly to miniaturization of the separation column and to the use of the single-pump system. The mass detection limit becomes important when the amount of sample available is limited.

On the other hand the concentration sensitivity of this system, *e.g.*, 33 $\mu$g/ml $\beta$-cyclodextrin, is much worse than the reported[12] value, *e.g.*, 1.0 $\mu$g/ml. However, the concentration sensitivity can be improved by a factor of ten because the injection volume can be increased up to 0.2 $\mu$l without significant loss of column efficiency. Moreover, the concentration sensitivity of micro HPLC can be improved by the use of the precolumn enrichment method[24], in which the sample volume is not generally limited if the enrichment conditions are carefully selected.

In order to take advantage of this system, two problems must be overcome in the future. First, the alkyl-modified silica employed in this work was not resistant to alkaline solution. The precision of the retention time and the peak area were poor under the conditions in Fig. 1, due to the short life of the separation column and instability of phenolphthalein. This is because a mobile phase with an high pH was used. The relative standard deviations of the retention time and peak area for four successive measurements under the conditions in Fig. 1 were 3.0 and 8.1% for $\gamma$-cyclodextrin and 4.9 and 4.8% for $\beta$-cyclodextrin, respectively. The efficiency of the separation column deteriorated during operation for 1 week. When the pH of the mobile phase was decreased to around 11, the colour intensity of the effluent from the column decreased in comparison with the original one, probably because the silica material consumed the alkali in the mobile phase, leading to a decrease in the pH value and to a decrease in the colour intensity. Therefore, a packing material which is resistant to alkaline solution must be selected. Fortunately, research on the improvement of the stability of alkyl-modified silica packings in alkaline solution is being

carried out in this field and materials suitable for this purpose will be available in the near future.

In addition, another strategy for this problem is to use the postcolumn mixing method as reported in a previous work on micro HPLC of bile acids[25]. In this case we compromise with a slight deterioration in the mass detection limits, but the precision and accuracy will be substantially improved.

Secondly, the background was gradually decreased and could not be stabilized. This drift of the baseline was due to the instability of the phenolphthalein solution. Cyclodextrins can form inclusion complexes with various compounds. Other reagents which are more stable than phenolphthalein should be found.

The indirect photometric detection based on perturbation of the partitioning of the visualization agent is another strategy to overcome these problems which is being investigated in this laboratory.

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