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Journal of Chromatography A, 746 (1996) 103–108

JOURNAL OF CHROMATOGRAPHY A

Analysis of partially methylated cyclodextrins by subcritical fluid and liquid chromatography

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Received 11 January 1996; revised 25 March 1996; accepted 26 March 1996

Abstract

Liquid and subcritical chromatographic analyses using evaporative light scattering detection were used to investigate the composition of various commercial dimethylated β-cyclodextrins (β-CD). The chromatographic fingerprints better depict the complexity of each mixture than the degree of substitution. The elution order is reversed when subcritical fluid chromatography is used instead of LC on apolar stationary phases (TMS and phenyl columns). Concerning the RPLC analysis of methylated β-CD, a phenyl-bonded silica column offers selectivities that are different from those obtained using a classical C_{18}-bonded silica column. With regard to the more complex mixtures, SubFC allows one to obtain richer methylated β-CD fingerprints than does LC.

Keywords: Cyclodextrins, methylated; Heptakis (2,6-di-O-methyl)-β-cyclodextrin; Carbohydrates

1. Introduction

Methylated β-cyclodextrins show very high solubilities in both water and organic solvents compared to their parent β-cyclodextrin (β-CD) and have a high capacity for including guest molecules of different sizes [1]. The formation of inclusion complexes changes a variety of physical, chemical or biological properties of the guest molecule, e.g. it increases solubility and bioavailability of low water-soluble substances. Consequently, they have been used in various fields, such as in the manufacture of cosmetics, food technology, pharmaceutical industry and in the production of agrochemicals. Moreover, cyclodextrins are often used as chiral discriminating agents in order to recognize numerous enantiomeric compounds in capillary electrophoresis (CE) [2], gas chromatography (GC) [3] and liquid chromatography (LC) [4]. Dimethyl β-cyclodextrin (DM-β-CD) seems to be the most useful drug carrier in biomedical fields [5].

However, the analysis as well as the high-purity isolation of these cyclodextrins is an actual problem. Methods previously used for the analysis of partially alkylated cyclodextrins include thin-layer chromatography [6,7], GC [6,8,9], high-performance liquid chromatography (HPLC) [6–10] and CE [11]. Supercritical fluid chromatography (SFC) or subcritical fluid chromatography (SubFC) offers selectivities that are different from the LC ones, and has the advantage of shorter analysis times. Therefore, there is great interest in investigating the methylated CDs by SFC or SubFC. As CDs are uncharged and demonstrate no appreciable UV absorbance, the LC

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0021-9673/96/$15.00
PII S0021-9673(96)00327-5

methods described so far are essentially based on refractive index (RI) detection [8,10]. Evaporative light scattering detection (ELSD), which is also a universal detection method, is more sensitive compared with RI and is compatible with the elution gradient. It proved to be a good choice for analytical studies of mono-, di- or trisaccharides by LC or SubFC [12–15], but it has never been used for CD analysis.

The aim of this preliminary work was to investigate the composition of four commercial samples of heptakis (2,6-di-O-methyl)-β-CD using isocratic SubFC and LC with ELSD.

2. Experimental

2.1. Apparatus

SubFC analyses were conducted with a model SF_3 Gilson apparatus (Villiers Le Bel, France), a Rheodyne (Berkeley, CA, USA) Model 7125 injector with a 20-μl sample loop, a CROCO-CIL column oven (CIL-Cluzeau, S^{te}.-Foy-la-Grande, France) and a Zorbax TMS (250×4.6 mm I.D.) column purchased from DuPont (Wilmington, DE, USA). The column temperature was 41°C. The use of a high percentage of modifier means that the conditions might not really be above the critical point, therefore we would call the chromatography performed here subcritical fluid chromatography (SubFC).

LC was carried out using a Varian (Palo Alto, CA, USA) Model 2510 solvent delivery pump, a Rheodyne Model 7125 injector with a 20-μl sample loop and a Zorbax Phenyl (250×4.6 mm I.D.) column purchased from DuPont.

Detection in both SubFC and LC was performed with an ELSD Sedex 45 Model (in LC) and Sedex 55 Model (in SubFC) (Sedere, Alforville, France). Concerning SubFC, a specific interface directly connected to the Gilson regulation pressure valve was used; the ELSD settings were as follows: photomultiplier, 7; evaporative temperature, 50°C; air pressure, 0.5 bar; nebulizer temperature, 75°C. In LC, ELSD settings were: photomultiplier, 7; evaporative temperature, 50°C; air pressure, 2.2 bar. Data were processed using Shimadzu (Kyoto, Japan) Model CR 5A integrators.

2.2. Reagents

Carbon dioxyde (B50 grade) was purchased from Air Liquide (Paris, France), methanol (Hipersolv grade, BDH) was from Prolabo (Paris, France), acetonitrile (RS for LC) was from Carlo Erba (Milan, Italy), pyridine (HPLC grade) was from Fluka (Buchs, Switzerland) and water was from Stalabo (Cooperation Pharmaceutique Française, Melun, France).

Acetonitrile–water (50:50, v/v) was used as the injection solvent in LC and methanol was used in subFC.

β-Cyclodextrin W 7 (β-CD) was purchased from Wacker (Lyon, France) and heptakis (2,3,6-tri-O-methyl)-β-cyclodextrin (TM-β-CD) was from Sigma (St. Louis, MO, USA).

Four different commercial dimethyl-β-CDs mixtures (A, B, C and D) were studied. They were characterized by an average degree of substitution (DS) as follows: A (DS=2.18); B (DS=2.10); C (DS=2.01) and D (DS=1.80).

Sample B was used in order to obtain pure heptakis(2,6-di-O-methyl)-β-cyclodextrin. From this mixture, three partially methylated β-CD derivatives (I, II and III) corresponding to the peaks 1, 2, 3, respectively, of chromatogram B (Fig. 2), were isolated by LC on a Zorbax Phenyl column with acetonitrile–water as the mobile phase. The spectrum of derivative I, obtained on a 500 MHz Bruker NMR, is identical to the ^1H spectrum of heptakis(2,6-di-O-methyl)-β-CD published by Yamamoto et al. [16].

3. Results and discussion

3.1. Subcritical fluid chromatography

Separation of different sugars (mono-, di- and trisaccharides) has already been successfully studied using SubFC, on various stationary phases, i.e., apolar (C_{18} or TMS) bonded silica [17] and polar (CN, Diol or NO_2) bonded silica [15]. As methylated β-cyclodextrins consist of seven glucopyranose units in which several hydroxyl groups are methylated, they could also be analyzed using SubFC.

For this preliminary study, a TMS-bonded silica

column was selected because it gave good results for the separation of glycolipids and sugars [18]. Analysis of polar compounds such as carbohydrates and cyclodextrins requires either addition of a polar modifier to the carbon dioxide fluid or derivatization of solutes which increases their solubility in carbon dioxide. The addition of a modifier compatible with ELSD was chosen due to the fact that derivatization of the solutes was not convenient. Methanol is commonly used in SubFC as a modifier to increase the elution power of the mobile phase and to improve peak shape through deactivation of active silanol sites on the surface of the stationary phase.

Fig. 1 shows the SubFC elution pattern of TM-β-CD and of three commercial DM-β-CDs on a Zorbax TMS column with a CO_2–methanol–water–pyridine (80:18.1:1.8:0.1, v/v) mobile phase.

As shown in Fig. 1, TM-β-CD had the shorter retention time (t_R) and was eluted before heptakis(2,6-di-O-methyl)-β-CD (which had the same elution time as peak 3 of B). The underivatized β-CD was not eluted under these conditions. The more methylated the CD was, the shorter the elution time was. Methylated CDs were eluted mainly in order of increasing polarity. These results are in good agreement with those obtained for the SubFC analysis of sugars [17]. The retention mechanism of methylated CDs using a TMS-bonded column and a

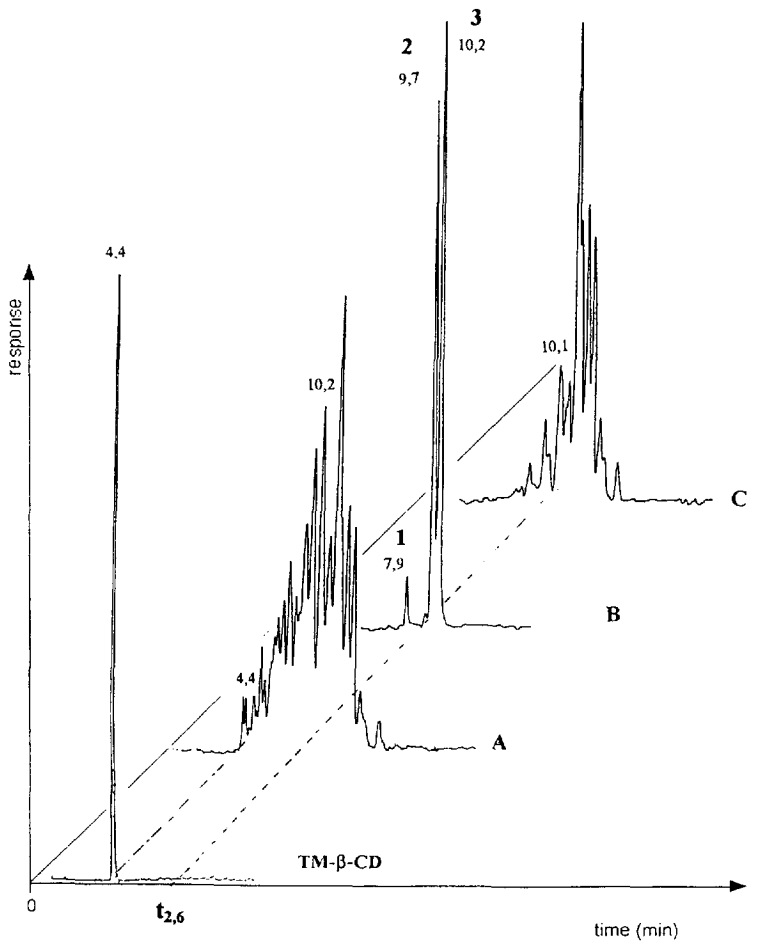

Fig. 1. SubFC separations of TM-β-CD, β-CD and three DM-β-CDs on Zorbax TMS (250×4.6 mm I.D.) with an evaporative light scattering detector. Mobile phase consisted of CO_2–methanol–water–pyridine (80:18.1:1.8:0.1, v/v); flow-rate, 3 ml min^{-1}; pressure, 168 bar. Solutes: TM-β-CD; A (DS=2.18); B (DS=2.10); C (DS=2.01). $t_{2,6}$=retention time of heptakis(2,6-di-O-methyl)-β-CD.

carbon dioxide-polar modifier eluent is close to the "normal-phase" process.

The three commercial DM-β-CDs did not exhibit the same elution profiles (Fig. 1). The complexity of these chromatographic fingerprints can be explained by the non-selective methylation at the 2, 3 and 6 positions of each glucose unit. We found commercial mixtures with different composition characterized by an average DS value (degree of substitution or the average number of methyl groups per anhydroglucose unit).

On the one hand, A (DS=2.18) had the largest distribution. The t_R value of the first peak corresponds to the t_R of TM-β-CD. On the other hand, B (DS=2.10) presented the simplest profile. Chromatographic separation shows that the degree of methylation is not an adequate criterion to illustrate the complexity of the partially methylated mixtures. A degree of substitution of approximately two (DS= 2.01 for C) did not mean that the sample consisted of pure heptakis(2,6-di-O-methyl)-β-CD.

Fig. 1 shows that samples A, B and C (which have a degree of methylation higher than two) all contained a chromatographic peak with the same retention time as heptakis(2,6-di-O-methyl)-β-CD. Among the four DM-β-CDs, D, which had the lowest degree of methylation (DS=1.8), and underivatized β-CD were not eluted under the chromatographic conditions used. This result was in good agreement with the low DS value obtained and confirms that D was less methylated (and therefore more polar) than the other samples.

In conclusion, SubFC with a TMS column and ELSD is a suitable method for DM-β-CD analysis. The behaviour of other stationary phases for the separation of DM-β-CDs will be published later.

SubFC provides characteristic fingerprints for each commercial DM-β-CD sample studied. Interpretation of these fingerprints permits a quick estimation of the composition of methylated mixtures.

3.2. High-performance liquid chromatography

Investigations of methylated and partially methylated cyclodextrins by RPLC have previously been reported by Koizumi et al. [6], Kubota et al. [10], Schomburg et al. [8] and Deege et al. [9]. Up to now, analysis was achieved on C_{18}-bonded silica columns. The aim of our study was to use another analytical system in order to obtain different selectivities and to achieve shorter analysis times. Phenyl-bonded silica columns were used successfully.

Fig. 2 shows the LC elution pattern of underivatized β-CD and four commercial DM-β-CDs on a Zorbax Phenyl column with an acetonitrile–water mobile phase.

Under these chromatographic conditions, underivatized β-CD, which is the highly polar compound, was eluted after the void volume (V_0=1.8 ml), and TM-β-CD, which is the most hydrophobic compound with all of its hydroxyl groups methylated, was not eluted. Heptakis(2,6-di-O-methyl)-β-CD, which presented intermediate polarity, was eluted after β-CD in the same elution time as peak 1 of B. Therefore, the t_R value increased with the total number of methyl groups. As a matter of fact, methylation caused the CDs to become more hydrophobic. Our preliminary study proves that the retention order on Zorbax Phenyl firstly depends on the molecular hydrophobicity. In LC, the elution order of CDs on Zorbax Phenyl, a moderately apolar stationary phase, is similar to the order noticed on C_{18}-bonded silica [9]. Moreover, the elution order is reversed in SubFC and LC (Fig. 1B and Fig. 2B) with apolar stationary phases (TMS and Phenyl).

Fig. 2 depicts chromatographic profiles of four different DM-β-CDs. The complexity of the partially methylated β-CD samples depends on the methylation procedure. Sample D, with the lowest degree of methylation (DS=1.80), had the shortest elution time. It was a less methylated mixture than the other three DM-β-CDs and it had only a low chromatographic peak intensity at the t_R value of heptakis(2,6-di-O-methyl)-β-CD.

The chromatographic profiles of C and A were similar. They presented an asymmetrical and large peak at a retention time of about 17 min, which shows that they consisted of many products. The complexity of those components was proven by the SubFC analysis which offers better efficiency for those samples.

Moreover, we observed good selectivity for the analyses of D and B. The chromatographic profiles of B were comparable in SubFC and LC systems, but

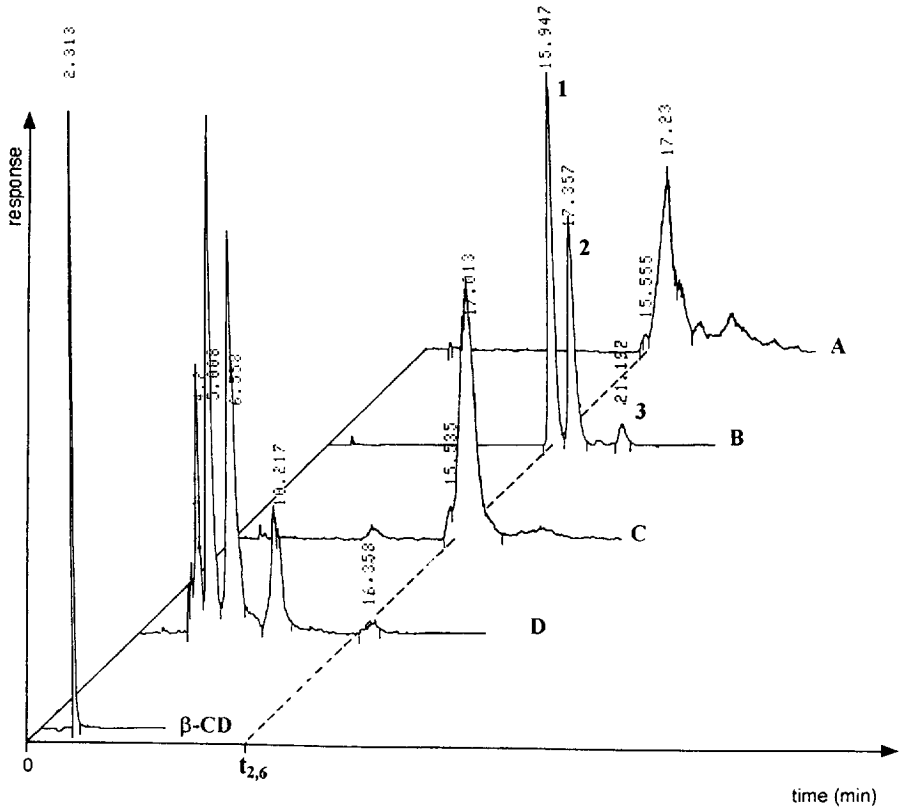

Fig. 2. LC separation of β-CD and four DM-β-CDs on Zorbax Phenyl (250×4.6 mm I.D.) with a light-scattering detector. Mobile phase, acetonitrile–water (33:67, v/v); flow-rate, 1 ml min^{-1}; pressure, 118 bar. Solutes: β-CD; A (DS=2.18); B (DS=2.10); C (DS=2.01); D (DS=1.80). $t_{2,6}$=retention time of heptakis(2,6-di-O-methyl)-β-CD.

a reversed elution order for the three peaks was observed.

Therefore, the LC system we proposed was not adequate for all the DM-β-CDs. The chromatographic systems must be carefully adjusted by varying both the stationary and mobile phases to the very different hydrophobicities of the various methylated CD species. The selectivities of the LC system used must be optimized for each DM-β-CD. Contrary to the LC method, SubFC proved to be efficient for the analysis of DM-β-CDs except for D, which was not eluted. LC and SubFC systems are therefore complementary for the analysis of partially methylated CDs.

In short, only B, which has the simplest SubFC and LC fingerprint, can give easier access to the pure products. The LC system was selected for the most efficient semi-preparative isolation of B. Moreover, the resolutions were better when using the LC system than when the SubFC system was used.

4. Conclusion

Heptakis(2,6-di-O-methyl)-β-CD was obtained with other methylated homologues in varying amounts, regardless of the method of β-CD methylation. Each mixture composition can be successfully verified by using LC or SubFC with ELSD detection.

This paper reports the first investigation where the analysis of cyclodextrins was carried out using SubFC. SubFC and LC provide characteristic fingerprints for each commercial DM-β-CD. These fingerprints better depict the complexity of each mixture

than do the DS values. For the more complex mixtures, SubFC allows one to obtain richer methylated β-CD fingerprints than does LC. SubFC can be used favourably as a complementary technique in the analysis of cyclodextrins. Using LC, it has been possible to isolate and purify heptakis(2,6-di-O-methyl)-β-CD from the simpler mixtures.

Obtaining pure partially methylated β-CD (e.g. heptakis(2,6-di-O-methyl)-β-CD, hexakis(2,6-di-O-methyl)–TM-β-CD) can be made possible using a combinatin of SubFC and LC techniques. It is of great interest to isolate symmetrical and asymmetrical chemically modified cyclodextrins in order to investigate the complex properties of these compounds.

Acknowledgments

This work, conducted by I. Caron and A. Salvador as part of their Ph.D. Theses, was supported by D.G.R.T. and Conseil Régional de la Région Centre (France), respectively.

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