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Determination of cyclodextrins in serum by reversed-phase chromatography with pulsed amperometric detection and a membrane reactor

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Abstract: A high-performance liquid chromatographic method has been developed for the determination of cyclodextrins (CDs) in serum. The method involves solid-phase extraction of CDs, separation on a C_{18} reversed-phase column using a mixture of water, tetrahydrofuran and methanol as an eluent, eluent pH modification with a cation-exchange membrane reactor surrounded by 1.5 M sodium hydroxide solutions, and pulsed amperometric detection (PAD) with a gold working electrode. The solid-phase extraction on a C_{18} bonded-silica column was effective for removing the PAD sensitive components in serum. The calibration graphs constructed by internal standard method were linear over the range 6.25–200 pmol of CDs in serum. The detection limits for CDs were about 5 pmol at a signal-to-noise ratio of 3.

Keywords: *Cyclodextrin determination; pulsed amperometric detection; reversed-phase chromatography; membrane reactor.*

Introduction

Recently, pulsed amperometric detection (PAD) with a gold working electrode for carbohydrates was developed by Rocklin and Pohl [1]. PAD is very sensitive not only for reducing aldoses and ketoses but also non-reducing sugars such as xylitol and sucrose [2–11], but has the disadvantage of its separation mode being limited to a strongly basic anion-exchange column with a highly alkaline eluent. In previous papers [12, 13], we reported a high-performance liquid chromatographic (HPLC) method for the determination of carbohydrates. The method involves separation by size-exclusion, normal-phase or reversed-phase mode, modification of the eluent pH to alkali using a membrane reactor and strong alkaline solutions, and PAD.

As CDs have unique properties such as enhancing the bioavailability or improving stability of drugs, they have been used as additives to pharmaceutical preparations. For the biopharmaceutical and pharmacokinetic studies, a sensitive assay method would be required to measure their levels in biological fluids. Previously [13], we reported that PAD of CDs in serum required sample pretreatment in the assay at the sub-pmol or pmol level. This paper deals with a sensitive HPLC procedure based on PAD for the determination of CDs in serum combined with solid-phase extraction on a C_{18} bonded-silica column. Also, improvement in eluent composition is proposed requiring little polishing of the working electrode.

Experimental

Reagents and materials

The CDs and control serum (Control Serum I) were obtained by Wako Pure Chemicals (Osaka, Japan). Methanol and tetrahydrofuran of HPLC grade were obtained from Nacalai Tesque (Kyoto, Japan). Water prepared by a NANOpure II unit (Barnstead, Boston, MA, USA) was used for the preparation of the sample and the eluent.

The cation-exchange membrane (AMMS-1) was obtained by Dionex (Sunnyvale, CA, USA).

Chromatography

The HPLC system was composed of a Model 4000i pump and a pulsed amperometric detector (PAD-1) (both from Dinonex) equipped with a gold working electrode and a silver–silver chloride reference electrode. The stationary phase used was a YMC ODS S-5

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silica (5 μm, Yamamura Chemical, Kyoto, Japan) packed into a 100 × 4.6 mm i.d. stainless steel tubing. The guard column (30 × 4.6 mm i.d.) packed with the same materials was used together with the main column. The eluents used were as follows: eluent A, water including 0.4% tetrahydrofuran and 0.5% methanol for the determination of β-CD in serum; eluent B, water including 0.175% tetrahydrofuran and 0.5% methanol for the determination of γ-CD in serum. The flow rate was maintained at 0.6 ml min^{-1}. The separation was carried out at 35°C using a TU-310 column oven (Japan Spectroscopic, Tokyo, Japan). A C-R6A Chromatopac (Shimadzu, Kyoto, Japan) was used for integrating and recording chromatograms. A sodium hydroxide solution at a concentration of 1.5 M was used as the eluent pH modifier, and delivered to a cation-exchange membrane (AMMS-1) at a flow rate of 1 ml min^{-1} by pressurizing a reagent reservoir with about 2.5 psi nitrogen. As reported previously [13], the applied pulse potentials and duration times for detection used were as follows: E_1 = 100 mV (t_1 = 120 ms); E_2 = 600 mV (t_2 = 120 ms); and E_3 = −800 mV (t_3 = 300 ms). The PAD response time was set at 1 s.

Preparation of serum samples

A known amount of β-CD or γ-CD was dissolved in human control serum. To 500 μl of the serum sample, a 50 μl aliquot of aqueous α-CD solution (4 nmol ml^{-1}) was added as internal standard. The serum samples were applied to a Baker-10SPE C18 column (J.T. Baker, Phillipsburg, NJ, USA), which is pre-activated by washing with 6 ml each of methanol and water. The column was washed twice with 2 ml of water, and then the CDs were eluted twice with 2 ml of 20% methanol. The eluate was evaporated to dryness, and the residue was reconstituted with 500 or 250 μl of the HPLC eluent used for the separation. The solution was filtrated with a 0.45-μm membrane filter (Biofield, Tokyo, Japan). A 50-μl portion of the filtrate was loaded onto the column.

Results and Discussion

Separation and detection

In a previous paper [13], we reported an HPLC method for the determination of CDs and branched CDs with pulsed amperometric detection followed by eluent alkalization using a membrane reactor and a strong alkaline solution. One disadvantage of the method is that the working electrode surface must be polished once or twice a month to prevent a decrease in sensitivity and increases in baseline noise and drift. This is due to the use of an eluent containing acetonitrile as an organic modifier, as reported previously [13]. Thus, we attempted to find an eluent system which can offer good sensitivity without polishing. When a mixture of water and tetrahydrofuran was used as the eluent, the PAD response was lower than that with a mixture of water and acetonitrile and the baseline noise was larger. When a mixture of water, methanol and tetrahydrofuran were used, the response and baseline noise was almost the same as that with a mixture of water and acetonitrile. This could be attributed to the complete mixing of the eluent and pH modifier by addition of methanol. Thus, 0.5% methanol was added to the eluent and the retention of CDs was adjusted by changing the concentration of tetrahydrofuran. In this eluent system, the polishing of the working electrode was not necessary for 5 or 6 months.

Pretreatment of serum samples

A preliminary study on PAD detection of CDs in serum followed by ultrafiltration of serum samples revealed that sample pretreatment in the assay should be done at the pmol level [13]. Thus, the solid-phase extraction of CDs from serum samples on a C_{18} bonded-silica column was examined. Under the optimum extraction conditions described in the Experimental section, the absolute recoveries of α-, β-, and γ-CDs were 92, 89 and 90%, respectively. Since γ-, α- and β-CDs were eluted in that order in the eluent system used, α-CD was used as internal standard for the determination of β- and γ-CDs in serum. For the assay of α-CD, γ- or β-CD could be used as internal standard.

Under the optimum extraction and separation conditions, chromatograms of β- and γ-CDs in serum together with α-CD, the internal standard, are shown in Figs 1 and 2. They were well separated from the serum components at the sub-pmol or pmol level by performing solid-phase extraction, especially removing PAD active compounds in serum such as amino acids, sulphur compounds and/or OH-bearing compounds. Since few interference

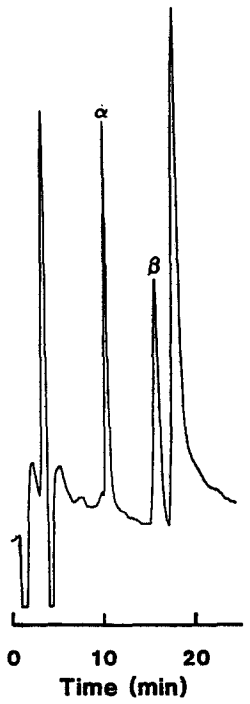

Figure 1
Chromatogram of β-CD in serum. α-CD (200 pmol/50 μl) was used as the internal standard. The injected amount corresponded to 100 pmol of β-CD. Detection was by PAD at 300 nA full scale. For chromatographic conditions, see Experimental section.

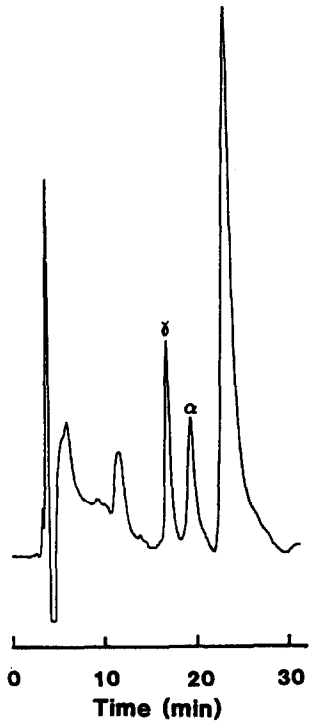

Figure 2
Chromatogram of γ-CD in serum. α-CD (200 pmol/50 μl) was used as the internal standard. The injected amount corresponded to 100 pmol of γ-CD. Detection was by PAD at 300 nA full scale. For chromatographic conditions, see Experimental section.

peaks were observed after elution of the background components of serum at a retention time of about 20 min, the repeated injection was performed with a run time of 25 min for the determination of β- and γ-CDs in serum.

Precision, linearity and detection limits

Table 1 shows the precision of the assay of β- and γ-CDs in serum, under the HPLC conditions given in Figs 1 and 2, respectively. Figure 3 shows the calibration graphs of β- and γ-CDs obtained using the internal standard method. The graphs were linear with correlation coefficients above 0.999, and the lines passed through the origins. The detection limits for β- and γ-CDs were about 5 pmol at a signal-to-noise ratio of 3.

Table 1
Precision of the assay of cyclodextrins (CDs) in serum

Assay no.	Peak height ratio of β-CD to α-CD	Peak height ratio of γ-CD to α-CD
1	0.317	0.828
2	0.322	0.793
3	0.294	0.800
4	0.284	0.833
5	0.290	0.888
6	0.289	0.806
Mean	0.299	0.825
SD	0.016	0.035
RSD	5.35	4.22

Injected amounts of α-, β- and γ-CDs were about 200, 100 and 100 pmol, respectively.

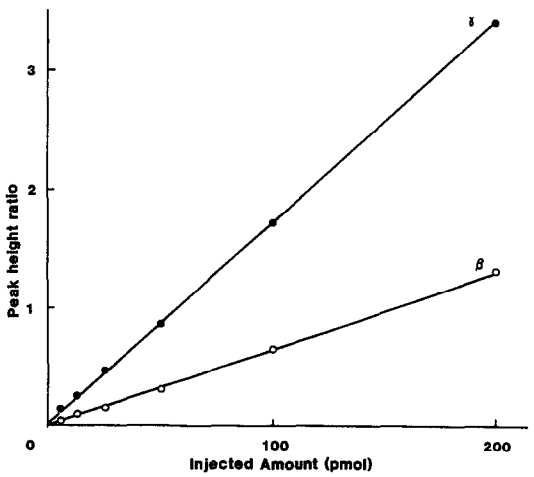

Figure 3
Calibration graphs of β- and γ-CDs in serum. α-CD (200 pmol/50 μl) was used as the internal standard.

In conclusion, CDs in serum can be sensitively determined by pulsed amperometric detection followed by solid-phase extraction.

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