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MICROANALYSES OF β-CYCLODEXTRIN IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Procedures for the micro-determination of β-cyclodextrin (β-CyD) in plasma were investigated by four methods using high-performance liquid chromatography (HPLC). In methods A and B, underivatized β-CyD was detected with a refractive index detector and determined by the absolute calibration graph method. An NH_2-bonded silica/acetonitrile—water system was used in A and a C_{18}-bonded silica/methanol—water system in B. In method C, the percarbanilate of β-CyD was separated on a C_8-bonded silica column with acetonitrile—water and determined using γ-CyD as the internal standard with a UV detector at 231 nm. In method D, the per[1-^{14}C]acetate of β-CyD was fractionated on a silica column with *n*-hexane—ethanol containing 1% of water and the radioactivity of each fraction was measured with a liquid scintillation counter. γ-CyD was used as the internal standard. Interfering plasma proteins were removed by centrifugal ultrafiltration with an MPS-1 micropartition system. Method B was superior to the other methods with respect to ease of sample preparation, sensitivity and time required for analysis. The cumulative amount of β-CyD in the mesenteric vein absorbed from the rat intestinal lumen after administration of phenobarbital—β-CyD complex in a closed loop method was determined by the use of method B.

INTRODUCTION

β-Cyclodextrin (β-CyD) forms inclusion complexes with barbiturates (BA) [1—5]. The complexation improves solubility of BA and results in increase in gastrointestinal absorption and, consequently, in enhancement of bioavailability of BA [6, 7]. Nevertheless, the mechanism of gastrointestinal absorption of the complexes is still uncertain. Generally, it has been assumed that only free drug coexisting with the drug—β-CyD complex at equilibrium in

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gastrointestinal fluids is absorbed on oral administration of the complex:

Drug + β-CyD ⇌ Drug—β-CyD

In our previous in vitro experiment of absorption using rat everted intestinal sacs [8], however, β-CyD was also detected in serosal fluid, and hence it was suggested that β-CyD may be absorbed from the gastrointestine in the form of a complex or in the intact form.

To establish whether β-CyD can be absorbed from the gastrointestine in in situ or in vivo absorption studies, the development of methods for the micro-determination of β-CyD in plasma is necessary. This paper reports four high-performance liquid chromatographic (HPLC) methods for determining β-CyD in plasma.

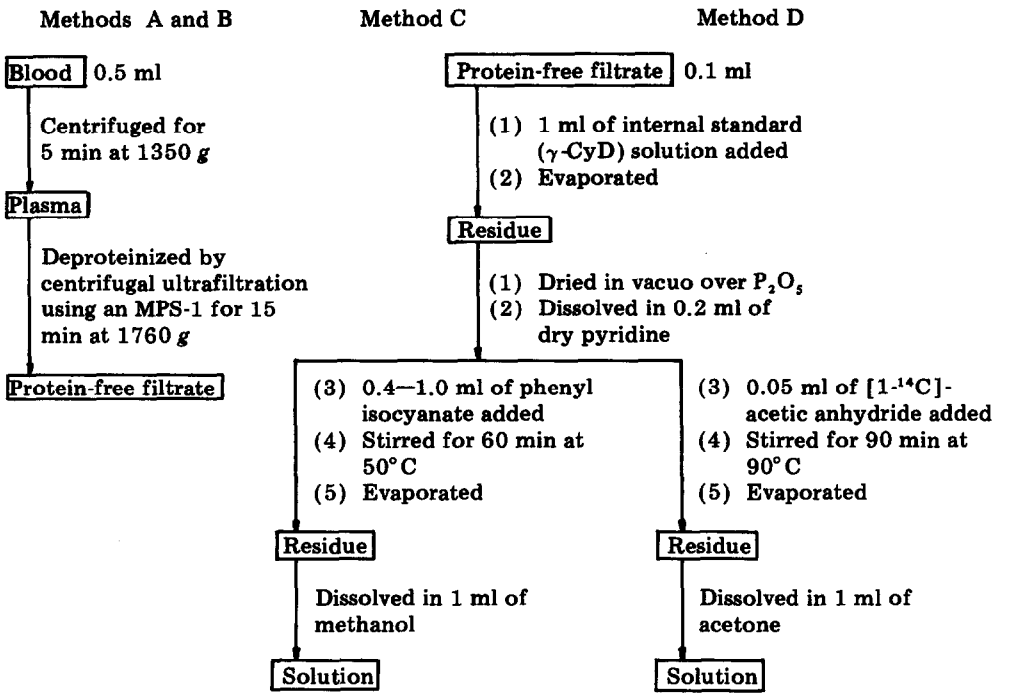
EXPERIMENTAL

Apparatus

A Tri Rotar SR-1 pump (JASCO, Tokyo, Japan) equipped with an SE-31 refractive index (RI) monitor (Showa Denko, Tokyo, Japan) and a VL-614 variable-loop injector (JASCO) was used for the determination of underivatized β-CyD. HPLC analyses of the β-CyD derivatives were conducted with a Model 6000A pump, a U6K injector (both from Waters Assoc., Milford, MA, U.S.A.) and a Uvidec-100III variable-wavelength detector (JASCO). The determination of [1-^{14}C]acetylated CyD was carried out using an LSC-700 liquid scintillation system (Aloka, Tokyo, Japan). The columns used were an ERC-NH-1171 (200 × 6 mm I.D.) (Erma Optical Works, Tokyo, Japan), a Hibar LiChrosorb RP-18 (250 × 4 mm I.D.) (Merck, Darmstadt, F.R.G.), a YMC-Pack A-212 (C_8) (150 × 6 mm I.D.) (Yamamura Chemical, Kyoto, Japan) and a Hibar LiChrosorb Si 60 (250 × 4 mm I.D.) (Merck). For the analysis of phenobarbital a semimicro liquid chromatograph equipped with a Familic-300S pump, an ML-425 injector, a Uvidec-100V detector and a μS-Finepak SIL C_{18} column (250 × 1.5 mm I.D.) (all from JASCO) was used. A Chromatopac C-RIA digital integrator (Shimadzu, Kyoto, Japan) was used to calculate peak areas. An MPS-1 micro-partition system (Amicon, Lexington, MA, U.S.A.) was used to remove plasma protein.

Materials

β-CyD was used after recrystallization from water, optical rotation, $[\alpha]_D^{18}$ +165.5°. γ-CyD, phenyl isocyanate (PHI) and acetic anhydride were of analytical-reagent grade. [1-^{14}C]Acetic anhydride (244 μCi/mg) and Scintisol EX-H as a scintillator were obtained from Amersham Japan (Tokyo, Japan) and Wako (Osaka, Japan), respectively. Phenobarbital (m.p. 176.3—176.8°C), of Japanese Pharmacopoeial standard, was purified by recrystallization. The organic solvents used for the preparation of mobile phase systems were of analytical-reagent grade and were freshly distilled before use. In all experiments distilled deionized water was used. The eluents were filtered through a 0.45-μm membrane filter and degassed.



Scheme 1. Preparation of sample solutions.

Preparation of sample solutions

Scheme 1 shows the preparation of each sample solution for HPLC analyses by the four methods. In methods A and B, 20 μl of protein-free filtrate was directly injected the chromatographic system. The amount of γ-CyD added as the internal standard (I.S.) was 2.5 or 10 μg in method C and 50 μg in method D. Pyridine used for derivatization of CyD, as a solvent having catalytic properties, was well dried by refluxing with anhydrous barium oxide and distilled before use. [1-^{14}C] Acetic anhydride (250 μCi) was used after dilution with 1 ml of acetic anhydride. At the end of the derivatization reactions excess of reagent and pyridine were removed by evaporation under reduced pressure. The methanol solution in method C and the acetone solution in method D were filtered through 0.45-μm membrane filters and subjected to HPLC.

Measurement of radioactivity in method D

Fractions of 1 ml were collected in vials and 6 ml of Scintisol EX-H were added to each vial for scintillation counting purposes. The radioactivities of [1-^{14}C] acetylated β-CyD and γ-CyD were measured as the sums of the radioactivities of corresponding fractions.

Proof of the structure of derivatized β-cyclodextrin

It was proved by elemental analyses of synthesized derivatives that all free hydroxy groups of β-CyD reacted with PHI or acetic anhydride.

Synthesis of β-CyD percarbanilate. β-CyD (1 g) was dissolved in 7.5 ml of dry pyridine at 50°C. PHI (4 ml, 42 mol.equiv.) was added and reaction was

carried out for 60 min at 50°C. Completion of the reaction was confirmed by thin-layer chromatography on a silica gel plate (Merck) with benzene—ethyl acetate (7:2) (R_F = 0.30). Most of the pyridine and PHI were evaporated under reduced pressure. The residue was dissolved in chloroform and washed with water. At that time most of the diphenylurea, the reaction product of water remaining PHI, was deposited between the water and chloroform layers. The chloroform layer was concentrated to dryness and purified by HPLC. The product was dissolved in hot isopropyl alcohol and reprecipitated on cooling, m.p. 216—217°C, $[\alpha]_D^{17}$ +71.0° (c = 1, pyridine) {lit. [9] m.p. 214—215°C, $[\alpha]_D^{25}$ +69.5° (c = 1, pyridine)}. Calculated for $C_{189}H_{175}O_{56}N_{21}$: C, 62.42; H, 4.85; N, 8.09%. Found: C, 62.46; H, 5.31; N, 8.00%.

Synthesis of β-CyD peracetate. β-CyD (1 g) was acetylated with acetic anhydride (5 ml) and dry pyridine (5 ml) for 90 min at 90°C, then the mixture was concentrated to dryness. The residual syrup was solidified by stirring with ice—water overnight. The amorphous powder obtained was crystallized from ethanol, m.p. 144—147°C. The crystals were convertible to other crystals having m.p. 211—212°C on recrystallization from a mixture of ethanol and methanol (1:1), and these two kinds of crystals showed the same optical rotation, $[\alpha]_D^{25.5}$ +123.5° (c = 2, $CHCl_3$) and had the same R_F value (0.72) on a silica gel plate with benzene—acetone (6:5), and were therefore polymorphic forms. Calculated for $C_{84}H_{112}O_{56} \cdot 2H_2O$: C, 49.12; H, 5.69%. Found: C, 49.01; H, 5.54%.

In situ intestinal absorption procedure

Male Wistar rats weighing between 250 and 260 g were fasted for 24 h prior to surgery and were anaesthetized with chloroform—diethyl ether (1:2). The small intestine was exposed by a midline abdominal incision, an intestinal segment (about 10 cm from ileal end) was cut and two glass cannulae were inserted at the both ends. The mesenteric arcades to adjacent portions were carefully tied off and the cannulae were ligated with silk suture. Heparin sodium solution was injected into the femoral vein. As a means of clearing the lumen, Krebs—Ringer solution warmed to 37°C was passed slowly from one end of the intestinal segment to the other. The remaining solution was carefully expelled from the lumen by means of an air pump and 0.5 ml of 0.1 *M* phenobarbital—β-CyD complex suspension in Krebs—Ringer solution, containing 1% CMC-Na and warmed to 37°C, was immediately introduced into the lumen. Both ends of the cannulae were connected to make a closed loop. The mesenteric vein was cannulated with an appropriate size of polyethylene tubing and venous blood was collected in centrifuge tubes in successive intervals, usually 2.5 min.

RESULTS AND DISCUSSION

Determination of underivatized β-cyclodextrin by HPLC with an RI detector

A few HPLC methods have been described for the separation of CyDs. A method using a cation-exchange resin column [Aminex 50W-X4 (Ca^{2+})] with water as the eluent [10] was applied at elevated temperature, but CyDs were very retarded, even at 90°C the retention time of β-CyD being over 30

min. Moreover, the relationship between peak area and β-CyD concentration, although demonstrating that the method can be used quantitatively, does not seem to be applicable to the micro-determination of β-CyD. HPLC analyses on an NH_2-bonded silica column (μBondapak-CH) with acetonitrile—water [11] and on a C_{18}-bonded silica column (Dextro-Pak) with methanol—water [12] are also thought to be unsuitable for the micro-determination of β-CyD, as the sharpness of the peaks in both chromatograms is insufficient. However, recent years have seen dramatic developments in the performance of columns for HPLC. In our previous study on the HPLC of cyclic (1→2)-β-D-glucans [13] excellent results could be obtained by using NH_2-bonded and C_{18}-bonded silica columns with 3—5 μm particles. Hence, HPLC analyses of β-CyD on these two types of columns were re-examined (methods A and B). In method A, an ERC-NH-1171 (3 μm) column was chosen as an NH_2-bonded silica column and acetonitrile—water (70:30) was used as the eluent. Method B was performed on a Hibar LiChrosorb RP-18 (5 μm) column with methanol—water (16:84). In order to minimize dilution of the sample, the removal of plasma protein was carried out by centrifugal ultrafiltration with an MPS-1 micro-partition system. Losses of β-CyD by this treatment were negligible. Typical HPLC results obtained by both methods for a standard β-CyD and a deproteinized plasma (blank) are shown in Figs. 1 and 2. In both chromatograms no peak in the plasma overlaps with that of β-CyD. Quantitative evaluation was carried out by

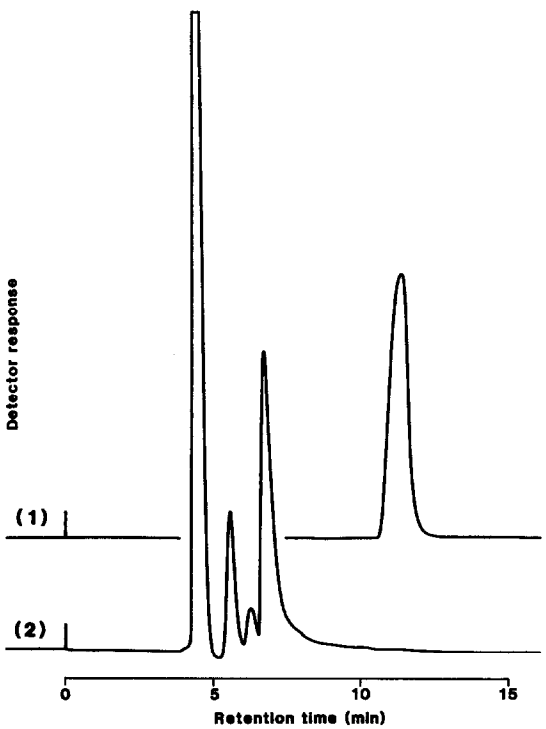


Fig. 1. Chromatograms of a β-cyclodextrin standard (0.2 μmol) (1) and a deproteinized plasma (20 μl) (2) on NH_2-bonded silica. Chromatographic conditions: column, ERC-NH-1171 (200 × 6 mm I.D.); eluent, acetonitrile—water (70:30); flow-rate, 1.0 ml/min; detector, Shodex RI SE-31 at $1.6 \cdot 10^{-4}$ RI units full scale; temperature, ambient.

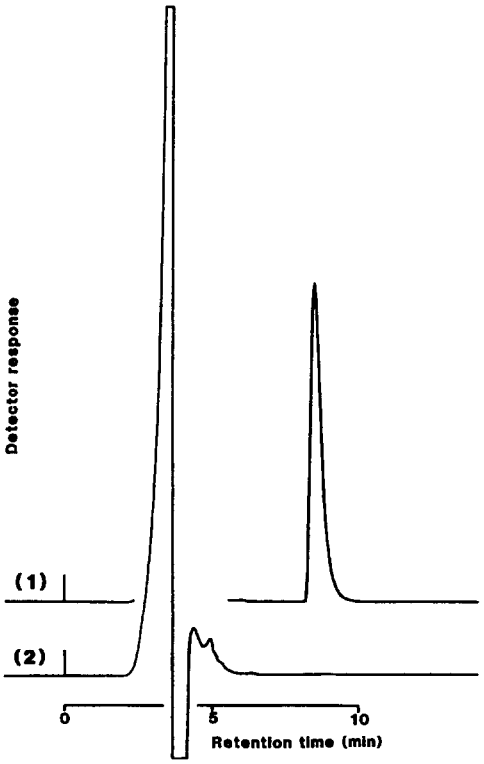


Fig. 2. Chromatograms of a β-cyclodextrin standard (0.06 μmol) (1) and a deproteinized plasma (20 μl) (2) on C_{18}-bonded silica. Chromatographic conditions: column, Hibar LiChrosorb RP-18 (250 × 4 mm I.D.); eluent, methanol—water (16:84); flow-rate, 0.6 ml/min; detector, Shodex RI SE-31 at $8 \cdot 10^{-5}$ RI units full scale; temperature, ambient.

TABLE I

CALIBRATION GRAPHS AND DETECTION LIMITS FOR THE FOUR METHODS

Method	Calibration graph			Detection limit for S/N* = 3 (nmol)
	Regression line	Correlation coefficient	Lower limit of determination (nmol)	
A	$y = 1.348x$	$r = 0.998$	0.4	0.09
B	$y = 1.913x$	$r = 0.999$	0.2	0.04
C	$y = 0.530x$	$r = 0.999$	0.2	0.04
D	$y = 0.153x + 0.247$	$r = 0.998$	0.9	0.20

*Signal-to-noise ratio.

the absolute calibration graph method using peak areas. The linearity of the detector response was investigated by injection of progressive dilutions of a β-CyD standard. Using method A the response was linear over the range 0.5—5 μg with the RI detector set at $0.25 \cdot 10^{-5}$ RI units full scale and over the range 4—40 μg at $2 \cdot 10^{-5}$, and using method B the linear range of the

detector response was 0.25—2.5 μg at $0.25 \cdot 10^{-5}$ and 2—20 μg at $2 \cdot 10^{-5}$. The regression lines and the correlation coefficients of the calibration graphs at higher sensitivity, the lower limits of determination and the detection limits at a signal-to-noise ratio of 3 are summarized in Table I. It is generally considered that RI monitoring is not suitable for micro-analyses. However, the use of a high-sensitivity RI detector together with a pump producing little pulsating flow allowed detection down to the 10^{-2} nmol level. Comparing methods A and B, the eluent in method B, methanol—water (16:84), offered a higher baseline stability of the RI detector at high sensitivity than that in method A, acetonitrile—water (70:30). Moreover, C_{18}-bonded phases are much more stable than NH_2-bonded phases.

Determination of phenyl isocyanate derivatized β-cyclodextrin by HPLC with a UV detector

Björkqvist [14] reported that the free hydroxy groups of saccharides and sugar alcohols react with PHI to yield very stable and strongly UV-absorbing derivatives, which possess good chromatographic properties in a reversed-phase system. He separated and determined some mono-, di- and trisaccharides, cello-oligomers up to octasaccharide and alditols on self-packed Spherisorb 5 ODS columns.

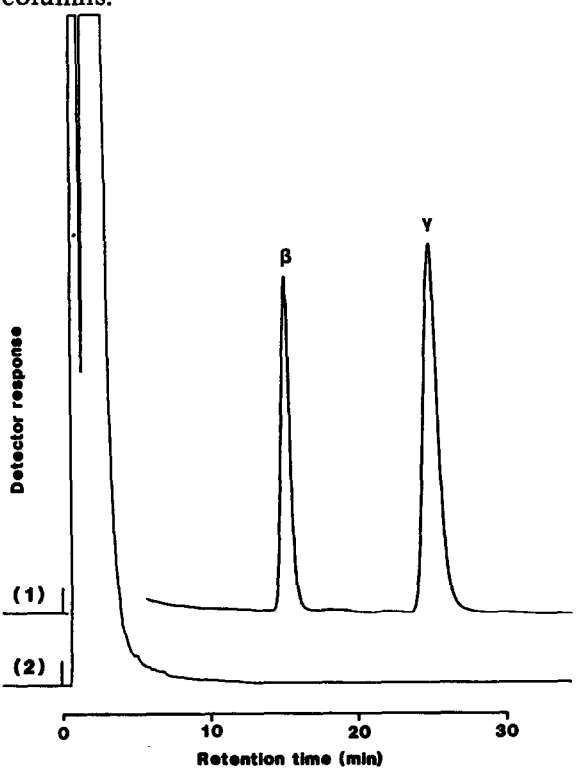


Fig. 3. Chromatograms of a phenyl isocyanate derivatized standard mixture of β-cyclodextrin (0.02 μmol) and γ-cyclodextrin (0.03 μmol) (1), and the blank (2) on C_8-bonded silica. Chromatographic conditions: column, YMC-Pack A-212 (150 × 6 mm I.D.); eluent, acetonitrile—water (89:11); flow-rate, 2.5 ml/min; detector, JASCO Uvidec-100III at 231 nm, 0.16 absorbance units full scale; temperature, ambient.

A modified method was applied to the determination of β-CyD. All free hydroxy groups of β-CyD reacted with PHI, as shown by elemental analyses of the synthesized derivative. Although Björkqvist [14] degraded the excess of PHI by addition of methanol, it turned out that most of the excess of PHI must be removed by evaporation under reduced pressure before addition of methanol, otherwise large amounts of the urethane derived from methanol and PHI interfere with the rapid analysis of β-CyD, because the urethane is eluted before the β-CyD derivative. To prevent the formation of diphenylurea, the reaction product of water and PHI, strictly anhydrous conditions should be maintained throughout the derivatization procedure. The internal standard method using γ-CyD was used for the determination. The β-CyD and γ-CyD percarbanilates were retained for a long period on C_{18}-bonded silica and a C_8-bonded silica column best fitted to determine β-CyD percarbanilate. Each of the CyD carbanilates yielded a single peak that did not overlap with peaks in the chromatogram of the blank (Fig. 3). The regression line and the correlation coefficient of the calibration graph obtained at 0.01 absorbance units full scale, the lower limit of determination, and the detection limit at a signal-to-noise ratio of 3 are shown in Table I.

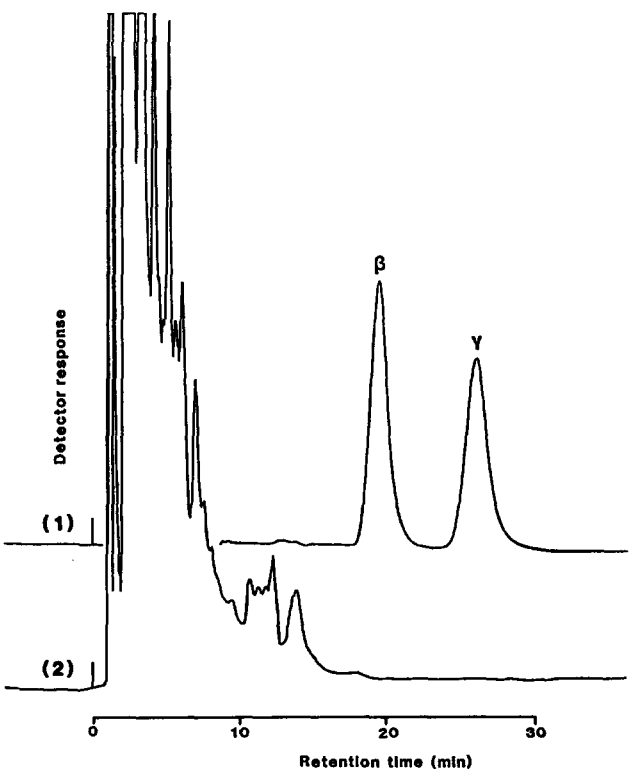


Fig. 4. Chromatograms of a peracetylated standard mixture of β-cyclodextrin (0.88 μmol) and γ-cyclodextrin (0.77 μmol) (1), and the blank (2) on silica. Chromatographic conditions: column, Hibar LiChrosorb Si 60 (250 × 4 mm I.D.); eluent, *n*-hexane—ethanol (73:27) containing 1% of water; flow-rate, 2.0 ml/min; detector, JASCO Uvidec-100III at 210 nm, 0.02 absorbance units full-scale; temperature, ambient.

Determination of [1-^{14}C]acetylated β-cyclodextrin with a scintillation counter after fractionation by HPLC

Derivatization of β-CyD with radioactive reagents should make this a very sensitive analytical technique. [1-^{14}C]Acetic anhydride was chosen as the radioactive reagent, as acetic anhydride readily reacts with all free hydroxy groups of CyD. First, the chromatographic conditions were investigated. By using a mobile phase consisting of *n*-hexane and ethanol, effective resolution of the acetates of β-CyD and γ-CyD, the I.S. and plasma components was achieved on a silica column. Tailing of peaks was prevented by addition of 1% of water to the eluent. Fig. 4 shows the elution profile of the mixture of peracetylated β-CyD and the I.S., γ-CyD, and a plasma sample treated with acetic anhydride in the same manner (blank). Quantitative analysis was carried out by fractionation of the eluate and scintillation counting of the fractions containing [^{14}C]acetylated β-CyD and γ-CyD. As shown in Table I, the calibration graph is sufficiently linear for quantitative analysis. The lower limit of determination and the detection limit are not very low, but could be lowered in theory by minimizing the dilution of [1-^{14}C]acetic anhydride with acetic anhydride. However, this is very costly and, moreover, the use of a reagent with high radioactivity should be avoided.

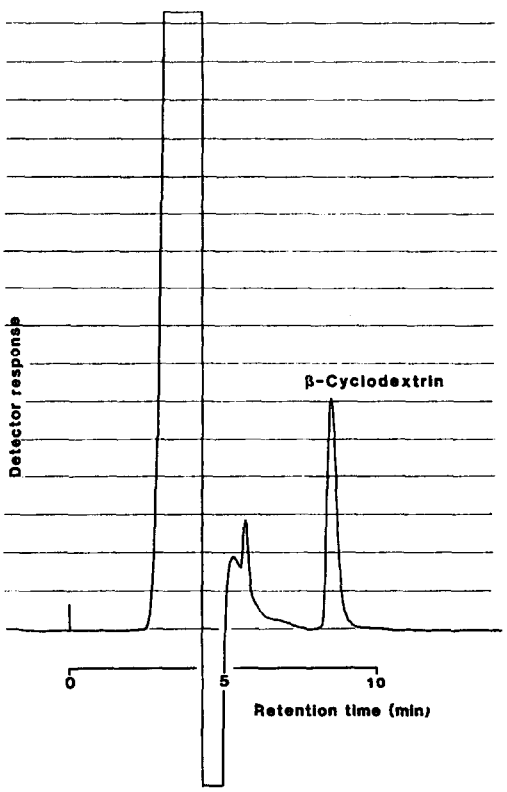


Fig. 5. Example of the determination of β-cyclodextrin in plasma collected during the second 2.5 min after administration of phenobarbital—β-cyclodextrin complex (50 μmol) in a closed-loop method. Chromatographic conditions: detector, Shodex RI SE-31 at $1 \cdot 10^{-5}$ RI units full scale; sample size, 20 μl; other conditions as in Fig. 2.

Determination of β-cyclodextrin in an in situ absorption study

Using method B, it is possible to detect 0.04 nmol of β-CyD. This result is comparable to the detection limit of percarbanilated β-CyD using UV detection (method C), and the lower limits of determination by both methods are the same. However, method B has the advantages of a shorter analysis time and no requirement for derivatization of β-CyD. Therefore, the amount of β-CyD in

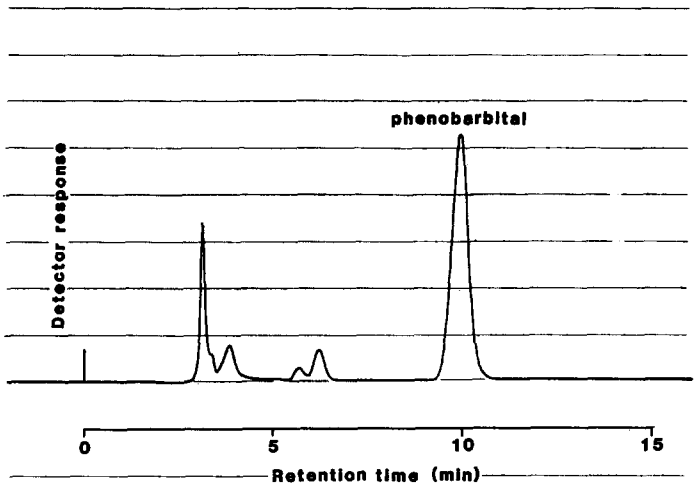


Fig. 6. Example of the determination of phenobarbital in plasma collected during the second 2.5 min after administration of phenobarbital—β-cyclodextrin complex (50 μmol) in a closed-loop method. Chromatographic conditions: column, μS-Finepak SIL C_{18} (250 × 1.5 mm I.D.); eluent, methanol—water (45:55); flow-rate, 0.1 ml/min; detector, JASCO Uvidec-100V at 220 nm, 0.64 absorbance units full scale; temperature, ambient; sample size, 3 μl.

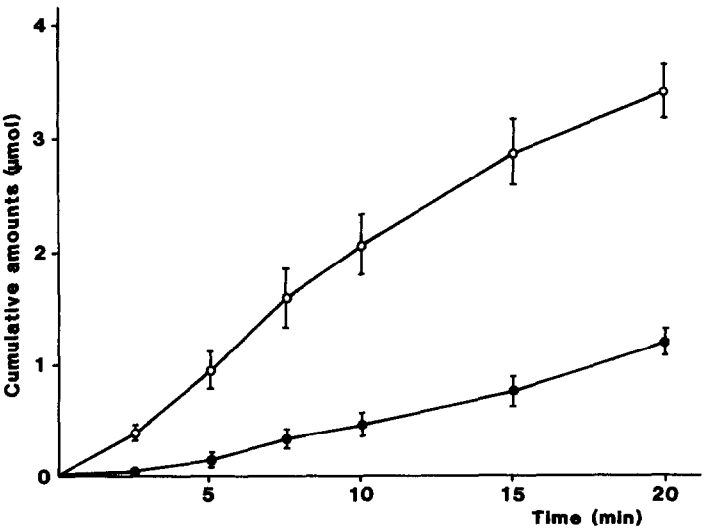


Fig. 7. Cumulative amounts of β-cyclodextrin (•) and phenobarbital (○) in mesenteric vein absorbed from rat intestinal lumen after administration of phenobarbital—β-cyclodextrin complex (50 μmol) in a closed loop method. Each point and vertical bar indicates the mean ± standard error for five rats.

the mesenteric vein absorbed from the rat intestinal lumen after administration of phenobarbital–β-CyD complex in a closed loop method was determined by the use of method B. For the purpose of comparison, phenobarbital in plasma was also determined by semimicro HPLC on a μS-Finepak SIL C_{18} (250 × 1.5 mm I.D.) column using a UV detector at 220 nm. Fig. 5 shows an example of the determination of β-CyD in plasma collected during the second 2.5 min after administration of the complex. Fig. 6 shows the determination of phenobarbital in the same plasma sample. As can seen in Fig. 7, although the rate of appearance of β-CyD in mesenteric blood, in situ, is slower than that of phenobarbital and the cumulative amount of β-CyD absorbed is smaller, β-CyD is undoubtedly able to be absorbed from the rat intestinal lumen.

In future work we shall carry out in situ absorption studies of β-CyD complexes and develop a method that is applicable to in vivo absorption studies.

REFERENCES

1 K. Koizumi and K. Fujimura, Yakugaku Zasshi, 92 (1972) 32.
2 A.L. Thakkar, P.B. Kuehn, J.H. Perrin and W.L. Wilham, J. Pharm. Sci., 61 (1972) 1841.
3 K. Koizumi, K. Mitsui and K. Higuchi, Yakugaku Zasshi, 94 (1974) 1515.
4 M. Otagili, T. Miyaji, K. Uekama and K. Ikeda, Chem. Pharm. Bull., 24 (1976) 1146.
5 T. Miyaji, Y. Kurono, K. Uekama and K. Ikeda, Chem. Pharm. Bull., 24 (1976) 1155.
6 K. Koizumi and Y. Kidera, Yakugaku Zasshi, 97 (1977) 705.
7 K. Koizumi, H. Miki and Y. Kubota, Chem. Pharm. Bull., 28 (1980) 319.
8 K. Koizumi and Y. Kubota, unpublished data.
9 I.A. Wolff and C.E. Rist, J. Amer. Chem. Soc., 70 (1948) 2779.
10 H. Hokse, J. Chromatogr., 189 (1980) 98.
11 B. Zsadon, K.H. Otta, F. Tűdős and J. Szejtli, J. Chromatogr., 172 (1979) 490.
12 K. Koizumi, Y. Okada, S. Horiyama, T. Utamura, M. Hisamatsu and A. Amemura, J. Chromatogr., 265 (1983) 89.
13 K. Koizumi, Y. Okada, T. Utamura, M. Hisamatsu and A. Amemura, J. Chromatogr., 299 (1984) 215.
14 B. Björkqvist, J. Chromatogr., 218 (1981) 65.