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DETERMINATION OF CYCLODEXTRINS IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH NEGATIVE COLORIMETRIC DETECTION USING POST-COLUMN COMPLEXATION WITH PHENOLPHTHALEIN

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SUMMARY

A rapid and sensitive high-performance liquid chromatographic method for the analysis of β- and γ-cyclodextrin in aqueous biological fluids such as plasma, urine, or tissue homogenate is described. The chromatographic system consists of a μBondapak Phenyl column as stationary phase and a mobile phase of water with 10% methanol. After post-column addition of an alkaline solution of phenolphthalein, negative colorimetric detection is used. The elution solvent and post-column reagent were mixed in a capillary tubing of 1.5 m (1.0 mm I.D.). Two methods of sample treatment are given, one for large (1.0 ml) and one for small (0.1 ml) sample volumes. Both methods were shown to be linear and reproducible. The detection limit for β-cyclodextrin was 1.0 μg/ml (0.77 nmol/ml). The method was used in the determination of some pharmacokinetic parameters of β-cyclodextrin in rats after intravenous injection.

INTRODUCTION

The unique qualities of cyclodextrins in various pharmaceutical areas have been widely acknowledged in recent years. Properties such as the enhancement of bioavailability or the improvement of stability of drugs have been described.

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Many reports about their use in various pharmaceutical dosage forms have been published during the past decade [1–4].

In spite of the fact that many papers have dealt with the parenteral application of cyclodextrins, and although a soluble powder for injections of prostaglandin-E1 stabilized with α-cyclodextrin is already on the market in Japan, only a few papers have discussed the bioanalysis of cyclodextrins [5,6]. The reason for this might be the difficulty of detecting cyclodextrins, because they show almost no UV absorption. Neither fluorescence detection nor electrochemical detection is a good alternative. Only refractive index detection can be used, but this method is generally not sensitive enough in the desired concentration range. Some workers solved the detection problem by using cyclodextrins labeled with a radioactive nuclide, such as carbon-14 [6].

For the separation of cyclodextrins by high-performane liquid chromatography (HPLC) several stationary phases have been described, e.g. amine-modified silica [5,7], alkyl-modified silica [5], vinyl alcohol polymer (Asahipak GS-320) [8], or cation-exchange resins such as Aminex 50W-X4 [9,10]. Water, or mixtures of water and organic solvents such as acetonitrile or methanol, have been used as mobile phases.

In the course of our present experiments concerning the biopharmaceutical properties of cyclodextrins, we needed a bioanalytical method for the determination of cyclodextrins for non-radioactive material. We therefore developed a rapid and sensitive method for the determination of cyclodextrin concentrations in biological samples by HPLC with negative colorimetric detection using post-column addition of an alkaline solution of phenolphthalein. The detection principle is based on the finding, described by Vikmon [11], that the decrease of colour intensity of phenolphthalein is proportional to the cyclodextrin concentration. The method was applied to the determination of some pharmacokinetic parameters of β-cyclodextrin in rats.

EXPERIMENTAL

Chromatographic system

The analyses were performed on a Waters liquid chromatograph (Waters Assoc., Milford, MA, U.S.A.), consisting of two 510 pumps, a U6K injector and a Model 440 absorbance detector. The detector was set at 546 nm and mostly operated at 0.1 a.u.f.s. with an offset of about 0.600 a.u.f.s.

A μBondapak Phenyl column (Waters Assoc., mean particle diameter 10 μm, 300×3.9 mm I.D.) was used at room temperature (ca. 20°C). The eluent was methanol–water (10:90). The flow-rate was 2.0 ml/min, resulting in a back-pressure of 21.2 MPa (3000 p.s.i.). The second pump was used to supply post-column reagent at a flow-rate of 2.0 ml/min, resulting in a back pressure of 1.1 MPa (150 p.s.i.). Between the tee connector and the detector a stainless-steel capillary tubing of 1.5 m (1.0 mm I.D., 1/16 in. O.D.) was assembled to improve mixing of both streams (Fig. 1). The post-column reagent was heated to 60°C.

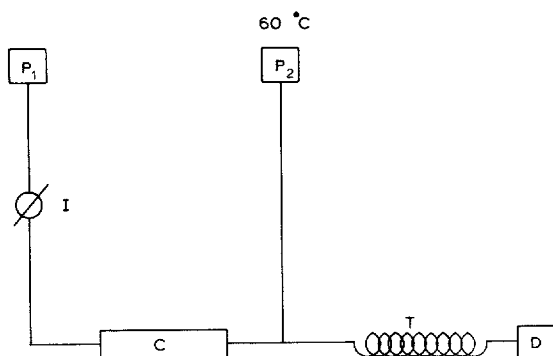


Fig. 1. Schematic diagram of the chromatographic system: P_1 = pump for mobile phase; P_2 = pump for post-column reagent; I = injection valve; C = column; T = capillary tubing; D = detector.

Materials

β-Cyclodextrin was kindly supplied by AVEBE, Veendam, The Netherlands. Both α- and γ-cyclodextrin were obtained from Sigma (St. Louis, MO, U.S.A.). Standard solutions of 5, 10, 50, 100 and 1000 μg/ml were made in water. The water used was deionized and distilled. A phenolphthalein stock solution was prepared by dissolving phenolphthalein in 96% (w/v) ethanol to a final concentration of 0.006 *M*. The post-column reagent was made by addition of 10.00 ml of the phenolphthalein stock solution to 990 ml of a 0.008 *M* sodium carbonate solution in water. The reagent was adjusted to pH 10.5 with a few drops of 1 *M* sodium hydroxide solution.

All chemicals were of reagent grade (Merck, Darmstadt, F.R.G.).

Methods

Two methods were used for plasma samples. In the first (method A) 250 μl of a trichloroacetic acid solution (20% in water) were added to 1.0 ml of plasma in a glass tube. After mixing for 30 s on a Vortex mixer and centrifugation for 5 min at 3000 *g*, the upper layer was poured into another tube and one drop of 4 *M* sodium hydroxide solution was added. The tube was shaken for a few seconds, and 250 μl were injected into the HPLC column. In the second method (method B) 100 μl of water were added to 100 μl of plasma. After shaking for some seconds, 50 μl of a trichloroacetic acid solution (20%) were added. After mixing and centrifugation the upper layer was poured into another tube and one drop of 1 *M* sodium hydroxide solution was added. The tube was shaken for some seconds, and the total solution (ca. 180 μl) was injected into the HPLC column. Peak heights were used to calculate β-cyclodextrin concentrations based on calibration curves prepared from spiked plasma samples.

Urine samples could be measured by injecting them directly into the column. In some cases the urine samples had to be diluted before injection. Tissue concentrations could be determined according to the following procedure. An aqueous homogenate of the tissue (2.5 g in 5 ml of water) was prepared by using an ultra turrax high efficiency dispenser. After centrifugation for 30 min at 3000 *g*, the supernatant was determined by method A.

Pharmacokinetic experiments

Three male Wistar rats (weight 420–450 g) were anaesthetized with urethane (1.0 g/kg given intraperitoneally). β-Cyclodextrin was dissolved in physiological saline at a concentration of 18 mg/ml. A dose of 25 mg/kg was injected intravenously (pineal vein). Blood samples of 250 μl were taken from the cannulated carotid artery at appropriate times and were immediately placed in ice. Plasma samples were prepared by centrifugation (for 10 min at 3000 g). These samples were analysed according to method B.

RESULTS AND DISCUSSION

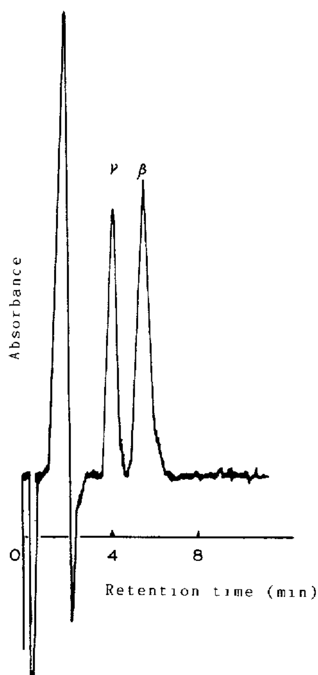
The detection method we used in our system is based on the decreasing effect of cyclodextrin on the UV–visible absorbance of phenolphthalein in aqueous alkaline solutions. The stability constant of the inclusion complex of β-cyclodextrin and phenolphthalein is 21 600 M^{-1} [11]. This large constant makes the detection system very sensitive and impervious to impurities, such as compounds that may affect the complexation of phenolphthalein with cyclodextrin. A second group of disturbing impurities are those that have an absorption at 546 nm and have the same retention time as cyclodextrin.

The chosen method of detection limits the composition of the elution solvents to water with at most 10% methanol, because in the presence of higher methanol concentrations (or other organic solvents) the formation of the complex between phenolphthalein and cyclodextrins is disturbed.

To find out which column was most suitable in our chromatographic system several columns were tested with elution solvents varying from pure water to water with 10% methanol. A Nucleosil 5 CN column (150×4.6 mm I.D.) and a LiChrosorb Diol column (150×4.6 mm I.D.) gave no retention at all with the solvents used. A Nucleosil 5 NO_2 (150×4.6 mm I.D.) and a Spherisorb-5 Hexyl column (150×4.6 mm I.D.) turned out to give retention times of 45 min or more. The μBondapak Phenyl column (300×3.9 mm I.D.) gave the best results. The retention time for β-cyclodextrin varied from 11.0 min with pure water as elution solvent to 5.1 min with water–methanol (90:10). This last solvent was used during the further experiments, because it gave the shortest analysis time.

A major problem was to obtain a stable baseline with low noise. The flow-rate of the post-column reagent had to be constant, and good mixing with the column eluate was required to obtain this feature both to get all cyclodextrin to form inclusion complexes with phenolphthalein, and to prevent baseline shifts caused by differences in the dilution of the post-column reagent. When the two solvents were simply mixed in the tee connector the baseline became jagged, fluctuating over a range equivalent to a peak height of at least 50 μg of β-cyclodextrin.

The 1.5 m (1.0 mm I.D.) capillary tubing with a volume of 1.18 ml turned out to give a high noise reduction. The mixed solvents have a flow-rate of 4.0 ml/min, so the residence time in the capillary is low and peak-broadening is negligible. Capillaries with other lengths and internal diameters were also tried, but the above-mentioned one gave the highest noise reduction and an acceptable peak-broadening.



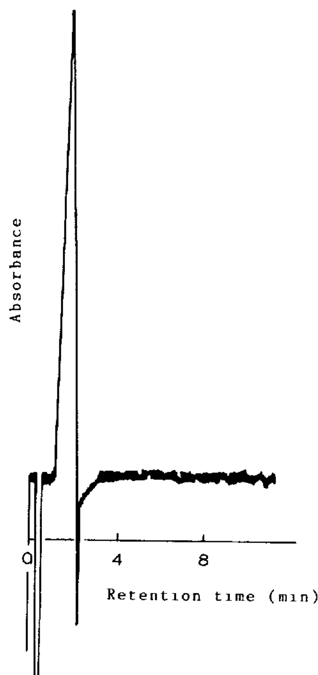


Fig. 2. Representative chromatogram of 100 μl of plasma spiked with 20 μg of β-cyclodextrin and 110 μg of γ-cyclodextrin (method B).

Fig. 3. Representative chromatogram of a blank rat plasma sample (method B).

The concentrations of phenolphthalein and sodium carbonate in the solvents after addition to the eluent are similar to those used by Vikmon [11]. We investigated other concentrations of phenolphthalein and sodium carbonate, but they did not give better signal-to-noise ratios. The reagent solution could be used for at least 48 h. To decrease the influence of room temperature variation and to prevent the development of gas bubbles, the phenolphthalein solution was heated to 60°C before addition to the eluent flow. This resulted in a temperature of ca. 40°C in the detector cell.

Fig. 2 shows a representative chromatogram of β-cyclodextrin and γ-cyclodextrin in plasma. As can be seen, the noise caused by irregularities in mixing of the two solvent flows has not completely disappeared, but is reduced sufficiently to allow measurements of low levels of cyclodextrins. Fig. 3 shows the chromatogram of a blank plasma sample. Fig. 4 shows the chromatogram of a rat plasma sample obtained 15 min after intravenous injection of 25 mg/kg β-cyclodextrin.

The retention time of γ-cyclodextrin (4.0 min) was shorter than that of β-cyclodextrin (5.1 min) so a mixture of β- and γ-cyclodextrins can be separated by this HPLC system (β-cyclodextrin $k'=11.4+0.5$). α-Cyclodextrin could not be detected with this sytem.

We found that a sample size of up to 1.0 ml did not disturb the peak shape. A β-cyclodextrin concentration of 1.0 μg/ml (0.77 nmol/ml) in plasma can be

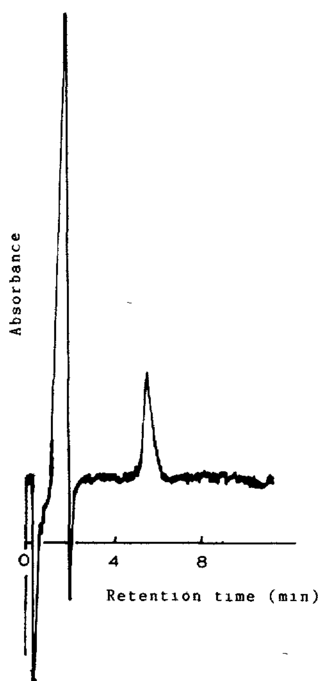


Fig. 4. Chromatogram of rat plasma obtained 15 min after intravenous injection of 25 mg/kg β-cyclodextrin (method B).

determined in this way without any preconcentration step. With 100-μl plasma samples analysed according to method B the detection limit is 10 μg/ml (signal-to-noise ratio 2.6). For method B the recovery from spiked plasma samples compared with standard solutions in water was 70.9%.

In the preparation of the calibration curve, method A was linear over the concentration range 2.5–50.0 μg/ml β-cyclodextrin and had an intercept of −0.04 μg/ml (correlation coefficient 0.9997). Method B was linear over the concentration range 5.0–40.0 μg/100 μl with an intercept of −0.06 μg/100 μl (correlation coefficient 0.9995). Further details on accuracy are shown in Tables I and II. Table III summarizes the results for the within-day and day-to-day precision of method B.

The method was less sensitive to γ-cyclodextrin. The detection limit of this substance was 5 μg/ml (3.8 nmol/ml) with a signal-to-noise ratio of 2.4 (method A). The fact that β- and γ-cyclodextrin can be separated on this column and both can be detected in our system offers the opportunity of using γ-cyclodextrin as an internal standard in the determination of β-cyclodextrin. Plasma samples could be stored at 4°C for 72 h without showing any decrease in β-cyclodextrin concentration.

The use of a solution of trichloroacetic acid for protein precipitation was the method of choice because it gives little dilution of the sample and clean chromatograms. A minor disadvantage of this procedure is the necessity to neutralize the low pH caused by the acid. Otherwise the absorbance of phenolphthalein

TABLE I

MEAN VALUES OF THE CONCENTRATIONS OF STANDARD SAMPLES IN PLASMA DETERMINED ACCORDING TO METHOD A ($n=6$)

β-Cyclodextrin added (μg/ml)	β-Cyclodextrin found (mean ± S.D.) (μg/ml)
0	0
2.5	2.40 ± 0.36
5.0	4.65 ± 0.84
10.0	9.85 ± 1.67
25.0	26.14 ± 1.20
50.0	49.49 ± 2.10

TABLE II

MEAN VALUES OF THE CONCENTRATIONS OF STANDARD SAMPLES IN PLASMA DETERMINED ACCORDING TO METHOD B ($n=6$)

β-Cyclodextrin added (μg/100 μl)	β-Cyclodextrin found (mean ± S.D.) (μg/100 μl)
0	0
5.0	4.35 ± 0.36
10.0	10.65 ± 0.82
20.0	20.42 ± 1.67
30.0	29.50 ± 0.96
40.0	40.06 ± 2.07

would decrease on account of the low pH and detection would be disturbed.

In the analysis of urine or tissue samples detection limits and recoveries are the same as for equal amounts of plasma. The accuracy and precision are of the same order of magnitude. Table IV shows the precision of the determination in urine.

Pharmacokinetic experiments

Fig. 5 shows the mean plasma disappearance curve of three rats after intravenous injection of 25 mg/kg β-cyclodextrin. The curve apparently consists of a

TABLE III

PRECISION OF METHOD B IN PLASMA

	β-Cyclodextrin (μg/100 μl)	C.V. (%)	*n*
Within-day	5	6.2	5
	10	5.8	5
	40	4.4	4
Day-to-day	5	8.3	5
	10	6.8	5
	40	5.1	4

TABLE IV

PRECISION OF THE DETERMINATON IN URINE

	β-Cyclodextrin (μg/ml)	C.V. (%)	n
Within-day	5	5.6	5
	10	5.3	5
	40	4.9	5
Day-to-day	5	6.6	4
	10	6.2	4
	40	5.3	4

distribution and an elimination phase, which can be characterized by the following parameters. Half-life of distribution phase, 9.5 min; primary volume of distribution, 138.1 ml/kg; half-life of elimination phase, 114 min; secondary volume of distribution, 394.9 ml/kg; clearance, 2.4 ml/min/kg; volume of distribution at steady state, 334.9 ml/kg.

It is obvious that the bioanalysis described above provides reliable information about the kinetic behaviour of β-cyclodextrin. Further studies are underway to determine the kinetic and toxicological properties of cyclodextrins after parenteral administration.

CONCLUSIONS

The method described in this paper provides a reliable and sensitive determination of β- and γ-cyclodextrin in biological fluids. The method is rapid and the chosen method of detection makes it very selective. When a negative colorimetric detection is used with strongly absorbing post-column reagents, mixing of both

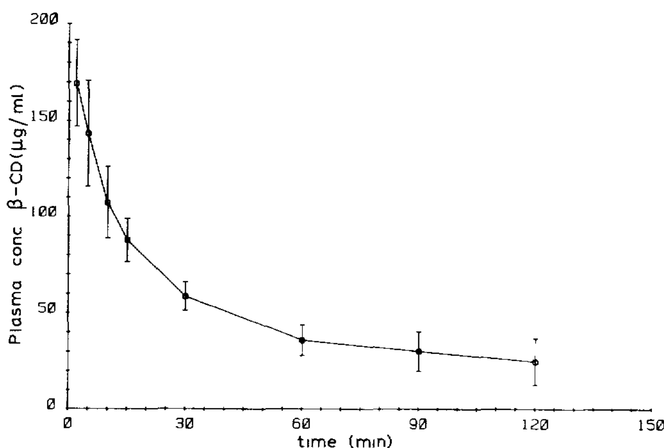


Fig. 5. Mean plasma disappearance curve of three rats after intravenous injection of 25 mg/kg β-cyclodextrin (β-CD); mean values ±S.D.

streams must be complete to prevent large baseline fluctuations. The bioanalysis was applied to pharmacokinetic studies of β-cyclodextrin, and the method clearly provides reliable information on its kinetic behaviour after intraveneous application.

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