A New Specific Enzyme Immunoassay Allowing an Efficient Pharmacokinetic Evaluation of γ-Cyclodextrin After Intravenous Administration to Rats

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Purpose. Because of its ability to form complexes with drugs, γ-cyclodextrin is of great potential value in pharmaceutical formulations. The biological fate of γ-cyclodextrin must therefore be considered in safety evaluation, using sensitive and specific methods applicable to biological fluids.

Methods. Antibodies were raised against γ-cyclodextrin, allowing the development of a new enzyme immunoassay. The analytical characteristics of this assay were evaluated. Rats were given a single intravenous 25 mg/kg dose of γ-cyclodextrin. Plasma and urine samples were collected and assayed.

Results. This new enzyme immunoassay was sensitive (limit of detection close to 94 pg/mL) and suitable for quantification of γ-cyclodextrin in urine and plasma after methanol extraction. The use of different linear and cyclic compounds demonstrated the high specificity of the assay. After i.v. administration, the concentration of γ-cyclodextrin rapidly decreased in the plasma while the molecule was probably distributed into the tissues. Although urinary elimination predominates, only 50% of the injected γ-cyclodextrin was recovered in urine, suggesting enzymatic degradation and/or tissular storage.

Conclusions. This assay may provide important information on the fate of γ-cyclodextrin inclusion complexes dedicated to drug-delivery using various modes of administration (oral, parenteral, transmucosal or dermal).

KEY WORDS: γ-cyclodextrin; enzyme immunoassay; acetylcholinesterase; pharmacokinetic analysis.

INTRODUCTION

Cyclomaltooligosaccharides (cyclodextrins or CDs) are natural molecules with the unique property of trapping organic compounds in their hydrophobic cavity. This process induces large variations in the solubility and stability of the "guest" molecules. Various applications for CDs have been considered in the pharmaceutical and food industries (for general reviews see 1–2). Cyclomaltooctaose (γ-CD) is the largest and most flexible natural cyclodextrin, and can therefore entrap larger "guests." Furthermore, this type of cyclodextrin is highly soluble in water and is weakly hemolytic, thus making it suitable for pharmaceutical formulations (3–4). However, information is required on its distribution before these potential applications are put into effect.

We have recently described a competitive enzyme immunoassay (EIA) for β-CD (5). Using a similar approach, we have developed a sensitive and specific assay which allows accurate quantification of γ-CD in biological fluids, as shown by the pharmacokinetic analysis of γ-CD in the rat after a single i.v. 25 mg/kg injection.

MATERIALS AND METHODS

Reagents and Buffer

Unless otherwise stated, all reagents were of analytical grade, from Sigma (St Louis, USA). All linear and cyclic oligosaccharides used are presented in Scheme 1. β-CD **2** and α-, γ-CD **1**, **3** were gifts from Roquette Frères (Lestrem, France) and Wacker (Germany), respectively. Octakis-(2,3,6-tri-O-methyl)cyclomaltooctaose **4** and Octakis-(2,3,6-tri-O-acetyl)cyclomaltooctaose **5** were gifts from Cyclolab (Budapest, Hungary). The key derivative mono (6-amino- 6-deoxy)-cyclomaltooctaose (6-monoamino-γ-CD, **6**) was obtained in three steps from the parent CD as described elsewhere (6). Compounds **7** and **8** were obtained by grafting the pertinent N-protected amino acids and subsequent deprotection with piperidine in DMF (6). The chemical and optical purities of compounds **6–8** were assessed by ^{1}H NMR, mass spectroscopy and chemical analysis. The mono(6-amino-6-deoxy)cyclomaltoheptaose derivative (6- monoamino-β-CD, **9**) was obtained as described (7–8). Maltooctaose **10** was from Fluka AG (Switzerland).

Acetylcholinesterase (AChE, EC 3.1.1.7) from the electric organs of the electric eel *Electrophorus electricus* was purified by affinity chromatography (9) and used in its G4 form for γ-CD labeling. AChE activity was measured using the colorimetric method of Ellman *et al.* (10).

All reagents used for immunoassays were diluted in the following buffer (EIA buffer): 0.1 M potassium phosphate buffer pH 7.4 containing 0.9% NaCl, 1 mM EDTA, 0.1% bovine serum albumin (BSA) and 0.01% sodium azide. For the washing steps, a 10 mM phosphate buffer containing 0.05% Tween 20 was used throughout.

Apparatus

Solid phase EIA was performed in 96-well microtiter plates (Immunoplate Maxisorb with certificate, Nunc, Denmark) using automatic Titertek microtitration equipment (washer, dispenser and reader) from Labsystems (Helsinki, Finland).

Antiserum Production

For the production of antibodies, 6-monoamino-γ-CD was covalently linked to bovine serum albumin (BSA). Glutaraldehyde (25% in water, 40 μL) was added to BSA (10.2 mg) and 6-monoamino-γ-CD (3.9 mg, 3 mmol) in 10 mL of 0.1 M pH 7.4 phosphate buffer. The reaction mixture was gently shaken for 18 h at 4°C in the dark and then dialyzed overnight against 0.1 M phosphate buffer (pH 7.4) at 4°C. Rabbits were immunized and boostered every month with 0.33 mg of immunogen

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according to the procedure described by Vaitukaitis (11) using complete Freund's adjuvant (DIFCO, Detroit, MI) and multiple subcutaneous injections. The rabbits were bled on a weekly basis after the first boosting step. Antisera were stored at 4°C in the presence of sodium azide (0.02% final concentration) and analyzed for titer and sensitivity using an enzyme immunoassay.

Preparation of the Enzymatic Tracer

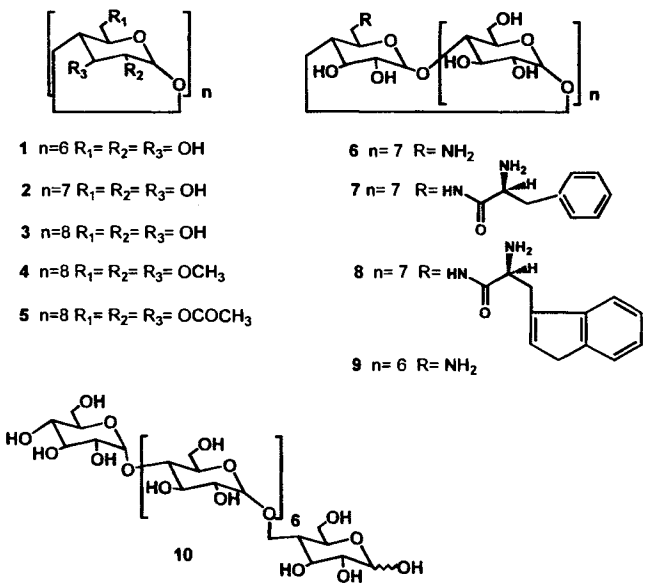
6-monoamino-γ-CD was covalently coupled to AChE using the heterobifunctional reagent N-succinimidyl-4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) as previously described for the preparation of β-CD-AChE (5) . This method involves the reaction of thiol groups previously introduced into 6-monoamino-γ-CD with maleimido groups incorporated into the enzyme. 6-Monoamino-γ-CD was thiolated by reaction of its primary amino group with N-succinimidyl-S-acetylthioacetate (SATA) in alkaline medium.

Competitive EIA Procedure

Competitive EIA was performed as described elsewhere for various molecules (12–14). Microtiter plates were coated with mouse monoclonal anti-rabbit IgG antibodies in order to ensure separation of bound and free moieties of the enzymatic tracer during the immunological reaction. Before use, the plates were washed with 0.01 M phosphate buffer (pH 7.4) containing 0.05% Tween 20. The total volume of the immunological reaction was 150 μL, each component (enzymatic tracer, diluted rabbit polyclonal antisera and cyclodextrin standard) being added in a 50 μL volume. The γ-CD-AChE enzyme conjugate was used at a concentration of 2 Ellman units/mL (for Ellman unit definition, see 13). The working dilution for the different rabbit antisera was previously determined by serial dilution experiments.

After 18-h incubation at 4°C, the plates were washed and the enzyme activity of the bound immunological complex was quantitated by addition of 200 μL of enzymatic substrate and chromogen (Ellman's reagent, 10) to each well. After 1 h of gentle shaking in the dark at room temperature, the absorbance at 414 nm in each well was measured. Results are given in terms of $B/B_0 \times 100$ as a function of the dose (logarithmic scale), B and B_0 representing the bound enzyme activity in the presence or absence of competitor, respectively. A linear log-logit transformation was used to fit the standard curve. Assay sensitivity was characterized by the dose of γ-cyclodextrin inducing a 50% reduction in the binding observed in the absence of competitor (B/B_0 50%). Non-specific binding represented less than 0.1% of the total enzyme activity. The minimum detectable concentration (MDC) was taken as the concentration of competitor inducing a significant decrease (3 standard deviations in B_0). All experiments were done in duplicate and quadruplicate for B_0.

For plasma and urine samples, a methanol precipitation procedure was performed before the assay. 4 volumes of cold methanol were added to the sample before centrifugation (2500 rpm, 10 min). The pellet was discarded and the supernatant dried under vacuum using a SpeedVac apparatus (Savant, Farmingdale, NY) before reconstitution with EIA buffer. Standard curves were plotted by spiking normal urine or plasma with known amounts of γ-CD, before performing the methanol precipitation, demonstrating more than 95% recovery of the molecule in the supernatant.



Scheme 1. Structure of the linear and cyclic maltooligosaccharides used in the present study. The different compounds correspond to: **1**, Cyclomaltohexaose (α-CD); **2**, Cyclomaltoheptaose (β-CD); **3**, Cyclomaltooctaose (γ-CD); **4**, Heptakis(2,3,6-tri-O-methyl)cyclomaltooctaose (per-Methyl-γ-CD); **5**, Heptakis(2,3, 6-tri-O-acetyl)cyclomaltooctaose (per-Acetyl-γ-CD); **6**, Mono(6-amino-6-deoxy)-cyclomaltooctaose (6-monoamino-γ-CD); **7**, Mono(6-L-phenylalanylamido-6-deoxy)cyclomaltooctaose (6-mono-Phe-γ-CD); **8**, Mono(6-L-tryptophanylamido-6-deoxy)cyclomaltooctaose (6-mono-Trp-γ-CD). **9**, Mono(6-amino-6-deoxy)cyclomaltoheptaose (6-monoamino-β-CD); **10**, Maltooctaose.

Animal Experiments

These investigations were carried out according to "The Principles of Laboratory Animal Care" (NIH publication #85-23, revised 1985). The experiments were performed on three 10-week-old (average weight 325 ± 60 g) male Sprague-Dawley rats (Iffa Credo, Les Oncins, France) which throughout the study had free access to water and to a commercial feed (DO4, UAR, Villemoisson, France). The rats were acclimatized for 4 days in individual metabolic cages before the experiments. The animals were anesthetized (one-third O_2, two-thirds NO_2 and 4% fluothane) and catheters were placed in the right femoral artery and vein, passed up into the abdominal vessels, and their free extremities exteriorized at the back of the neck. One day after surgery, the animals were given γ-CD in a single intravenous dose of 25 mg/kg in saline solution (49.6 mg/ml) by injection into the venous catheter. Blood (200 μl) was sampled at various times (0, 5, 10, 15, 20, 30 min, 1 h, 2 h, 4 h, 8 h, 24 h) at the arterial catheter and centrifuged. The resulting plasma was stored at −20°C and red blood cell pellets were reinjected through the venous catheter after dilution (1/2) with saline dilution. During the experiment, the rats were housed individually in glass metabolic cages which allowed separate

collection of urine and feces. Urine was collected 4, 8 and 24 h after injection.

Pharmacokinetic Evaluation

A specific computer program (Siphar from SIMED, Créteil, France), designed for non-linear regression analysis of pharmacokinetic data was used to fit plasma concentrations. Pharmacokinetic parameters were evaluated using a noncompartmental approach as described (15). The area under the plasma concentration curve (AUC) was determined using the trapezoidal lin-log rule and extrapolated to infinity. The elimination rate constant (ke) was determined by the decay of the plasma concentration over the last four values (between 30 min and 4 h). Clearance (Cl) was calculated from the dose divided by the AUC and the apparent volume of distribution was determined using the relation Cl/ke. Quantities eliminated in urine (Qe) were evaluated over 0–24 h. Urinary clearance was calculated on the basis of Clu = Qe/AUC. Values are expressed as the mean ± SD.

RESULTS

Assay Sensitivity

As previously described for β-cyclodextrin (5), the amino function of 6-monoamino-γ-CD constitutes a useful tool for specific antibody production. A very good antibody titer (above 1/100,000) was measured by EIA for the 3 rabbits from the first booster injection onwards. This indicates a strong immunogenic potency of the γ-CD-BSA immunogen. Surprisingly, the antisera titers remained virtually unchanged (>1/100,000) after the second booster injection for at least three months. Using the γ-CD-AChE tracer, we selected the best antiserum and optimized the dilution for the assay. A typical routine standard curve for an assay using antiserum (#L1124S8) at an initial dilution of 1/100,000 is shown in Fig.1. The sensitivity at B/B_0 50% was about 670 pg/mL (26 fmol/well), with a minimum detectable concentration close to 94 pg/mL (3.6 fmol/well). Assay precision was also very satisfactory as the coefficient of variation was below 10% in the 0.2–10 ng/mL range (see insert of Fig.1).

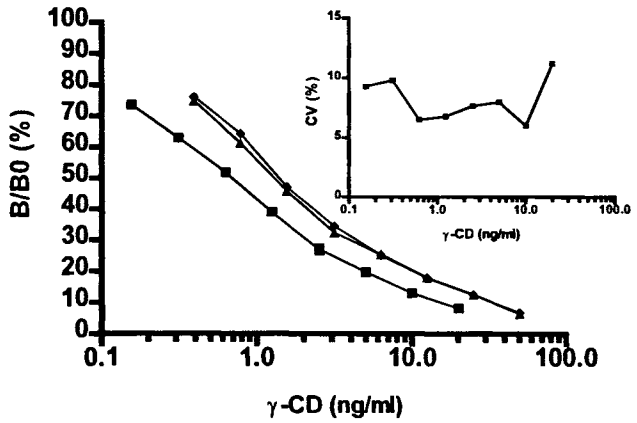


Fig. 1. Standard curve for γ-cyclodextrin enzyme immunoassay in EIA buffer (■) or in EIA buffer after extraction of rat plasma (▲) or urine (◆), (see Methods). Insert: "precision profile" for the present assay using data collected for the standard curve measurements performed in EIA buffer with 8 replicates for each dose of γ-CD. Assay precision is expressed as the coefficient of variation CV vs. the dose (logarithmic scale).

Assay Specificity

Antibody specificity was characterized by plotting standard curves using various derivatives of maltooligosaccharides. The results (Table 1) are expressed in terms of percentage cross-reactivity (CR) [(dose of γ-CD B/B_0 50%/dose of analog B/B_0 50%) × 100] (16).

γ-CD **3** as well as 6-monoamino-γ-CD **6** showed a similar full response, thus demonstrating that the amino group used for both the immunogen and the enzyme conjugate preparation is not involved in antibody recognition of the molecule. α-CD **1**, β-CD **2** and 6-monoamino-β-CD **9** were not recognized, demonstrating that the modifications introduced by the deletion of at least one D-glucose unit are sufficient to abolish antibody recognition. No significant cross-reactivity was noted in the assay of maltooctaose **10**, the linear analog of γ-cyclodextrin, thus showing that a cyclic conformation of the molecule is strictly required for a high antibody affinity. The epitope recognized by the antibodies therefore strictly corresponds to the cyclic maltooctaose core.

Different analogs of γ-CD (Table 1) were assayed. The methylated and acetylated derivatives **4** and **5** have substitutions at hydroxyl groups that can modify the recognition properties of cyclodextrins to various degrees. These compounds displayed very low cross-reactivity (CR < 0.1%) indicating that secondary hydroxyl groups play a key role in the recognition process since these derivatives are little different (in terms of molecular structure) from the parent cyclodextrins.

The effects of substitution at position C6 were also investigated. The phenylalanyl **7** and tryptophanyl **8** derivatives were recognized by the anti-γ-cyclodextrin antisera. It should be noted that NMR spectroscopy experiments have demonstrated that the phenylalanyl **7** derivative behaves as a self-inclusion complex in solution, the aromatic moiety of the amino acid being included in the cavity of its own γ-cyclodextrin carrier (6). This derivative is, however, recognized by the antibodies, indicating either that the self-inclusion process does not induce important changes in CD structure at the epitope level and/or that the binding of the antibodies reverses this process, resulting in the native CD structure.

Enzyme Immunoassay of γ-Cyclodextrin in Biological Fluids

We have investigated the analytical characteristics of the γ-CD immunoassay in biological fluids before performing the pharmacokinetic experiments. As previously observed for the 6-monoamino-β-CD enzyme immunoassay (17), when rat urine and plasma, spiked with known amounts of γ-CD, were assayed undiluted, we were confronted to a typical matrix effect. For instance, the binding of the tracer to the antibodies (B0) was currently significantly decreased and in most of the cases, the assay performed in crude plasma lacked of reproducibility associated with an important loss in sensitivity. A 100-fold dilution of these samples in EIA buffer always totally suppressed these interferences but consequently resulted in a lower absolute sensitivity directly linked to the dilution. We thus preferred to apply

Table 1. Relative Cross-Reactivity (%) with Different Compounds, Taking Cyclomaltooctaose as 100% Reference

Compound	CR (%)
Cyclomaltohexaose **1**	<0.1
Cyclomaltoheptaose **2**	<0.1
Cyclomaltooctaose **3**	100
Heptakis(2,3,6-tri-O-methyl)cyclomaltooctaose **4**	<0.1
Heptakis(2,3,6-tri-O-acetyl)cyclomaltooctaose **5**	<0.1
Mono(6-amino-6-deoxy)cyclomaltooctaose **6**	94
Mono(6-L-phenylalanylamido-6-deoxy)cyclomaltooctaose **7**	87
Mono(6-L-tryptophanylamido-6-deoxy)cyclomaltooctaose **8**	96
Mono(6-amino-6-deoxy)cyclomaltoheptaose **9**	<0.1
Maltooctaose **10**	<0.1

a simple methanol precipitation of the samples (see Methods), whereby assay sensitivity and reproducibility were recovered. Only a slight decrease in sensitivity was observed with extracted plasma and urine, with respective MDCs of 183 and 234 pg/ml (Fig. 1).

Pharmacokinetic Measurements of γ-CD

γ-CD was detected in plasma for up to 4 h (52 ± 21 ng/ml) after a single 25 mg/kg injection. Concentrations in urine remained at ≥10 μg/ml over the 8–24 h period. The plasma concentration curve and the urinary excretion of i.v. administered γ-CD are presented in Figs. 2 and 3, respectively. The main pharmacokinetic parameters are listed in Table 2. γ-CD rapidly disappeared from the plasma as demonstrated by a short half-life (28 min) and MRT (31 min). The apparent volume of distribution (Vd) exceeded the volume of blood and extracellular fluids (687 ml/kg), indicating that γ-CD is distributed into tissues and probably into the intracellular space. A substantial fraction of the injected γ-CD was eliminated in urine (52% at 24 h) with an apparent urinary clearance close to the renal glomerular filtration rate (18). The other half of the dose may be stored in tissues and/or undergo enzymatic degradation. To check these hypotheses, we performed a kinetic analysis of rat blood samples spiked with γ-CD, collected with heparin and incubated at 37°C (see methods). We observed a progressive decrease in γ-CD immunoreactivity, confirming the probable enzymatic degradation of the molecule (data not shown). Under these conditions, the half-life was close to 6 h.

DISCUSSION

We have described a new, sensitive, specific and reliable enzyme immunoassay for γ-cyclodextrin. This analytical method appears specific for γ-CD since neither α-CD and β-CD nor the linear analog (maltooctaose) were recognized, thus demonstrating that a well-defined macrocyclic structure involving 8 D-glucose units is required for a high antibody affinity. As previously shown for β-cyclodextrin, modifications at the primary hydroxyls do not affect recognition, while derivatives substituted at the secondary side are not detected. Taking into account previous data on β-CD antibodies, this suggests that the γ-CD molecule is probably recognized through the wider secondary hydroxyl rim. Assay sensitivity and accuracy in biological fluids are good after a methanol extraction step. In conditions where samples contain sufficient amounts of γ-CD, this time-consuming extraction step (which allows a recovery of the optimal characteristics of the assay in plasma or urine samples) can be replaced by a 100-times dilution in EIA.

In pharmacokinetic measurements in rats, this immunoassay afforded efficient and reliable analyses of blood γ-CD and urinary excretion of γ-CD after administration by intravenous bolus (25 mg/kg). This intravenously administered γ-CD disappears rapidly from the systemic circulation and elimination is primarily urinary. However, as previously described (19), a

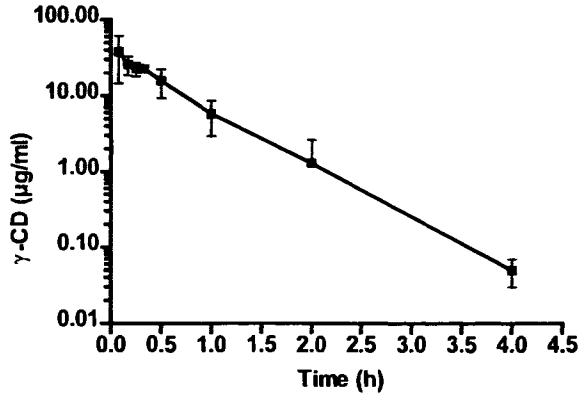


Fig. 2. Analysis of blood levels using γ-cyclodextrin immunoassay after i.v. administration of γ-cyclodextrin (25 mg/kg) to rats.

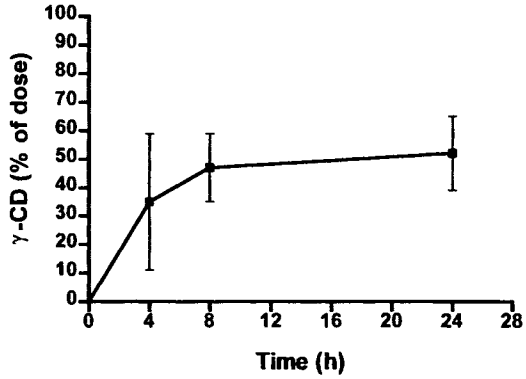


Fig. 3. Cumulative urinary excretion of γ-cyclodextrin after i.v. administration of γ-cyclodextrin (25 mg/kg) to rats.

Table 2. Main Pharmacokinetic Parameters of γ-CD After Intravenous Administration (25 mg/kg) to Rats

Rat		1	2	3	Mean ± SD
$T^1/_2$	min	26	33	25	28 ± 4
MRT	min	46	26	21	31 ± 13
Cls	$ml.min^{-1}.kg^{-1}$	14.7	19.7	16.2	16.9 ± 2.6
AUC 0-inf	$\mu g.ml^{-1}.h$	28.25	21.10	25.63	24.99 ± 3.63
Vdss	$ml.kg^{-1}$	546	930	586	687 ± 211
Qel 0–24 h	%dose	51	68	36	52 ± 12
Clu 0–24 h	$ml.min^{-1}.kg^{-1}$	7.9	12.6	5.8	8.8 ± 3.5

major part of the dose (close to 50%) appears to undergo enzymatic degradation in the blood stream, in contrast with α- and β-CDs, which are almost totally excreted intact in urine after intravenous administration. Nevertheless, since the apparent volume of distribution largely exceeds the volume of blood and extracellular fluids, deep compartments and storage in pools can not be excluded.

To our knowledge, this is the first full description of the pharmacokinetic analysis of γ-cyclodextrin after i.v. administration to the rat. This specific analytical method will provide information of major importance on the behavior and fate of cyclodextrin when used in pharmaceutical applications, irrespective of the mode of administration. Although the present study addressed the issue of the parenteral route of administration, the present assay may be used to examine γ-CD fate after other systemic (oral, transmucosal) or topical (dermal) routes of administration.

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