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A sensitive and specific enzyme immunoassay for cyclomaltoheptaose and some derivatives

Christophe Créminon [a,*], Florence Pilard [b], Jacques Grassi [a], Bruno Perly [b], Philippe Pradelles [a]

[a] *CEA, DRIPP, Service de Pharmacologie et d'Immunologie, Centre d'Etudes de Saclay F-91191 Gif s / Yvette, France*

[b] *CEA, DRECAM, Service de Chimie Moléculaire, Centre d'Etudes de Saclay F-91191 Gif s / Yvette, France*

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Abstract

Antibodies were raised against cyclomaltoheptaose (β-cyclodextrin), providing a highly sensitive enzyme immunoassay for β-cyclodextrin and some derivatives with a detection limit of 120 pg/mL. Investigations of cross-reactivities with a wide variety of linear and cyclic maltooligosaccharides demonstrate that the antibodies are highly specific for cyclomaltoheptaose and a number of derivatives. The epitope is probably located on the secondary hydroxyl groups of the rim side. This enzyme immunoassay is shown to be suitable to detect cyclomaltoheptaose in urine and in plasma.

1. Introduction

Owing to their ability of entrapping various small organic molecules into their hydrophobic central cavity, cyclomaltooligosaccharides (cyclodextrins or CDs) modify the physicochemical properties of the guest molecules by increasing their solubility and their stability, as well as in modifying their reactivity [1]. Numerous applications of these properties have been developed, particularly in agriculture and the food industry for the preservation of tastes and flavours, and in the cosmetic industry for the protection of included active substances against oxidation, photochemical reactions or losses by volatility. Pharmacological applications are based on an increase of the solubility of the active molecule. The interest in

* Corresponding author.

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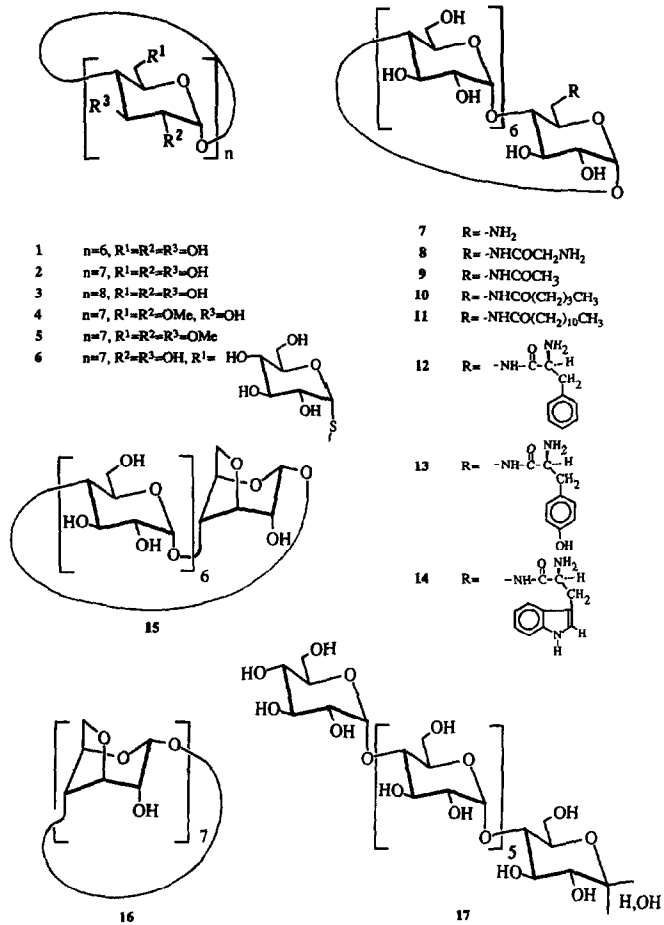
using cyclodextrins in the pharmaceutical industry rapidly grew after the discovery of parenterally safe derivatives which could permit intravenous administration of active molecules. The development of pharmaceutical applications requires sensitive and specific methods dedicated to quantitatively determine cyclodextrins and derivatives in biological material. At the present time, although colorimetric methods have been described [2], they suffer from severe limitations in terms of sensitivity and specificity. Using a synthetic derivative of cyclomaltoheptaose, we raised polyclonal antibodies in rabbits and developed a sensitive and specific competitive enzyme immunoassay (EIA) which could be a powerful and useful tool permitting both qualitative and quantitative measurement of intact β-cyclodextrin and derivatives in biological fluids.

2. Experimental

General methods and reagents.—Unless otherwise stated, all reagents were of analytical grade, from Sigma (St Louis, USA). All linear and cyclic oligosaccharides used in this study are presented in Scheme 1. Special care was taken to ensure that these compounds were of very high purity, ^{1}H NMR at high magnetic field being used as the major analytical technique to check for purity. Cyclodextrins were purchased from Roquette Frères (Lestrem, France). Heptakis(2,6-di-*O*-methyl)cyclomaltoheptaose (**4**) was obtained from Janssen (Beerse, Belgium) and recrystallized five times from MeOH to over 99% purity. Heptakis(2,3,6-tri-*O*-methyl)cyclomaltoheptaose (**5**) was from the same supplier and used as received. The key derivative 6-amino-6-deoxycyclomaltoheptaose [3] (**7**) was prepared from 6-*O*-*p*-tolylsulfonyl cyclomaltoheptaose [3,4] according to a modified procedure [5]. All other derivatives were prepared as described in the literature.

Pure acetylcholinesterase (AChE, EC 3.1.1.7) from the electric organs of the electric eel *Electrophorus electricus* was prepared according to ref 6 and its activity was measured as previously described [7]. The enzyme immunoassy (EIA) buffer was a 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. Solid-phase EIA was performed on 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-amino-6-deoxycyclomaltoheptaose (**7**) was covalently linked to bovine serum albumin (BSA) by use of glutaraldehyde. Glutaraldehyde (25% in water, 62 μL) was added to BSA (23 mg) and **7** (8 mg, 7 μmol) in 15.5 mL of 0.1 M pH 7.4 phosphate buffer. The mixture was gently shaken for 1 h at 22°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 each of immunogen according to the procedure described by Vaitukaitis [8] using complete Freund's adjuvant (Difco, Detroit, USA) and multiple subcutaneous injections. The rabbits were bled on a weekly basis after the first booster. Antisera were kept at 4°C in the presence of sodium



Scheme 1. Structure of the linear and cyclic maltooligosaccharides used in the present study.

azide (0.02% final concentration) and analyzed for titer and sensitivity using an enzyme immunoassay.

Preparation of the enzymatic tracer.—6-Amino-6-deoxycyclomaltoheptaose (7) was covalently coupled to AChE using the procedure previously described for haptens [9] and proteins [10]. Thiolation of 6-amino-6-deoxycyclomaltoheptaose was achieved by reaction of the amino group with *N*-succinimidyl-*S*-acetylthioacetate (SATA) in alkaline medium. To 7 (1.135 mg, 1 μmol) dissolved in 0.1 M (pH 8.5) borate buffer (1 mL), was added SATA (2.13 mg, 9.2 μmol) in anhyd DMF (50 μL). The mixture was allowed to react for 30 min at 25°C before purification with a Sep-Pak C_{18} cartridge(Waters, Milford, USA). The thiolated derivative was eluted with MeOH, freeze-dried and redissolved in degassed sodium phosphate buffer (0.1 M pH 6, 1.6 mL). A 1 M hydroxylamine hydrochloride solution (pH 7, 400 μL) was added and, 30 min later, the thiol content was determined colorimet-

rically (412 nm) after reaction with 0.5 mM 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) as described by Ellman [11]. Maleimido groups were introduced onto acetylcholinesterase (G4 form) by reaction with *N*-succinimidyl-4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) and subsequently purified as previously described [10]. Coupling of the thiolated cyclodextrin derivative (3 nmol) to maleimido–AChE (0.3 nmol G4-SMCC) was achieved by mixing the two compounds in 0.1 M phosphate buffer (pH 6, 0.275 mL). After 3 h reaction at 30°C, the enzyme conjugate was purified by gel filtration on a Biogel A 0.5 m column (90 × 1.5 cm) eluted with EIA buffer [9,10].

Competitive EIA procedure.—Competitive EIA was performed as described elsewhere [9,10]. Microtiter plates were coated with mouse monoclonal antirabbit immunoglobulins in order to ensure separation between the free and bound 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. β-CD–AChE enzyme conjugate was used at a concentration of 5 Ellman units/mL (for the definition of an Ellman unit, see ref 10). The working dilution for the different rabbit bleedings was previously determined by performing serial dilution experiments.

After an 18-h incubation period at 4°C, the plates were washed and the enzyme activity of the bound immunological complex revealed by addition of 200 μL of enzymatic substrate and chromogen (Ellman's reagent [12]) in each well. After 2 h of gentle shaking in the dark at room temperature, the absorbance at 414 nm in each well was measured automatically. Results are given in term 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–lin transformation was used to fit the standard curve. The sensitivity of the assay was characterized by the dose of β-CD inducing a 50% lowering of the binding observed in the absence of competitor (B/B_0 50%). Nonspecific binding represents less than 0.1% of the total enzyme activity. Finally, 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 made in duplicate and quadruplicate for B_0.

3. Results and discussion

Sensitivity of the assay.—Owing to the haptenic nature of β-cyclodextrin, preparation of an immunogen with a carrier protein was necessary to obtain antibody production. We used the glutaraldehyde method applied to 6-amino-6-deoxycyclomaltoheptaose [3] (**7**), to ensure covalent coupling between the amino group of the hapten and similar functionalities of the carrier molecule. The same amino group of **7** was used for a covalent coupling to acetylcholinesterase with a different cross-linker in order to ensure a full immunoreactivity of the enzyme

tracer and to avoid specific recognition of the spacer arm by antibodies. This was achieved by derivatization of the amino function by *N*-succinimidyl-*S*-acetylthioacetate leading, after treatment with hydroxylamine, to a thiolated derivative allowing an efficient coupling with maleimido–acetylcholinesterase. Since a covalent linkage of the same single amino group is used in both cases, the enzyme–oligosaccharide conjugate structurally mimics the immunogen and is perfectly recognized by the antibodies. Using this tracer, we have selected the best bleeding and the optimal antiserum dilution for the assay. A typical routine standard curve for an assay using antiserum (no. L1076S7) at 1/50 000 initial dilution is presented in Fig. 1. The sensitivity at B/B_0 50% is ca. 720 pg/mL (32 fmol/well) with a minimum detectable concentration close to 120 pg/mL (5.2 fmol/well). The precision of the assay is also very satisfactory as a variation coefficient of less than 9% was observed in the 0.2–50 ng/mL range (50–5000 pg/well) as shown in Fig. 1.

Specificity of the assay.—To characterize the specificity of the antibodies, standard curves were performed with various derivatives of maltooligosaccharides. The results are expressed in term of percentage of cross-reactivity (CR) [(dose of β-CD B/B_0 50%/dose of analogue B/B_0 50%) × 100] and are summarized in Table 1.

Cyclomaltoheptaose (**2**) as well as 6-amino-6-deoxycyclomaltoheptaose (**7**) showed a full cross-reactivity, thus demonstrating that the amino group used for both the immunogen and the enzyme conjugate preparation is not involved in the recognition of the molecule by the antibodies. Cyclomaltohexaose (**1**) and cyclomaltooctaose (**3**) (α- and γ-cyclodextrins, respectively) were poorly recognized. This demonstrates that the modifications introduced by the deletion or addition of one D-glucose unit are sufficient to abolish antibody recognition. The use of the linear analog of β-cyclodextrin, maltoheptaose (**17**) which exhibits a very poor cross-reac-

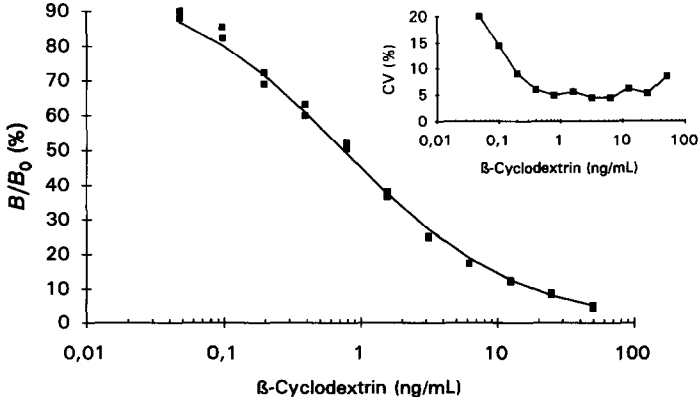


Fig. 1. Standard enzyme immunoassay titration curve for β-cyclodextrin. Insert: precision profile for the present assay using data collected for the standard curve with 8 replicates for each dose of β-CD. The precision of the assay is expressed as the coefficient of variation CV vs. the dose (logarithmic scale).

tivity, established that a macrocyclic structure of the molecule is strictly required for a high antibody affinity. Thus, the epitope recognized by the antibodies strictly corresponds to the cyclic maltoheptaose.

Different analogues of cyclomaltoheptaose (Table 1) were assayed. The first set (compounds **4**, **5**, **15**, and **16**) presents substitutions of hydroxyl groups that can modify the recognition properties of cyclodextrins to various degrees. The methylated derivatives (**4**) and (**5**) displayed a very low cross-reactivity (CR < 0.1%), indicating that hydroxyl groups play a key role in the recognition process since these derivatives are virtually not very different (in terms of molecular structure) from the parent cyclodextrins. A single intramolecular dehydration to a mono-3,6-anhydro derivative [13] (**15**) decreased the recognition by the antibodies (CR 24%) while a complete conversion into a heptakis(3,6-anhydro) derivative [14] (**16**) led to a totally abolished recognition (CR < 0.1%). In compound **16**, the conformation of all D-glucose rings is indeed reversed from the $^4C_1$ to $^1C_4$(D) chairs, leading to considerable variations of the overall molecular structure [14].

With a second set of derivatives, the effects of various substituents at one or several carbon C-6 atoms were investigated. As displayed in Table 1, all these compounds (**6–14**) are recognized by the anti-β-cyclodextrin antisera. Even the complete replacement of all primary hydroxyl groups by bulky thioglucosyl moieties, as in heptakis(6-*S*-α-D-glucopyranosyl-6-thio)cyclomaltoheptaose [15] (**6**), did not abolish recognition. Comparison of the cross-reactivities of these compounds with those observed for **4** and **5** led to the suggestion that the epitope could be localized onto the secondary hydroxyl rim of cyclomaltoheptaose.

Table 1
Relative cross-reactivity (%) obtained with compounds **1–17** taking cyclomaltoheptaose as 100% reference

Compound	B/B_0 50% (fmol/mL)	CR (%)
Cyclomaltohexaose (**1**)	877	< 0.1
Cyclomaltoheptaose (**2**)	0.7	100
Cyclomaltooctaose (**3**)	642	< 0.1
Heptakis(2,3-di-*O*-methyl)cyclomaltoheptaose (**4**)	> 10000	< 0.1
Heptakis(2,3,6-tri-*O*-methyl)cyclomaltoheptaose (**5**)	> 10000	< 0.1
Heptakis(6-*S*-α-D-glucopyranosyl-6-thio)cyclomaltoheptaose (**6**)	0.52	136
6-Amino-6-deoxycyclomaltoheptaose (**7**)	0.66	106
6-Deoxy-6-glycylamidocyclomaltoheptaose (**8**)	1.03	69
6-Acetamido-6-deoxycyclomaltoheptaose (**9**)	1.21	59
6-Deoxy-6-pentanoylamidocyclomaltoheptaose (**10**)	0.45	156
6-Deoxy-6-dodecanoylamidocyclomaltoheptaose (**11**)	0.78	91
6-Deoxy-6-L-phenylalanylamidocyclomaltoheptaose (**12**)	0.89	80
6-Deoxy-6-L-tyrosylamidocyclomaltoheptaose (**13**)	2.46	29
6-Deoxy-6-L-tryptophanylamidocyclomaltoheptaose (**14**)	0.64	111
$3^A,6^A$-Anhydrocyclomaltoheptaose (**15**)	3.04	24
Heptakis(3,6-anhydro)cyclomaltoheptaose (**16**)	778	< 0.1
Maltoheptaose (**17**)	1101	< 0.1

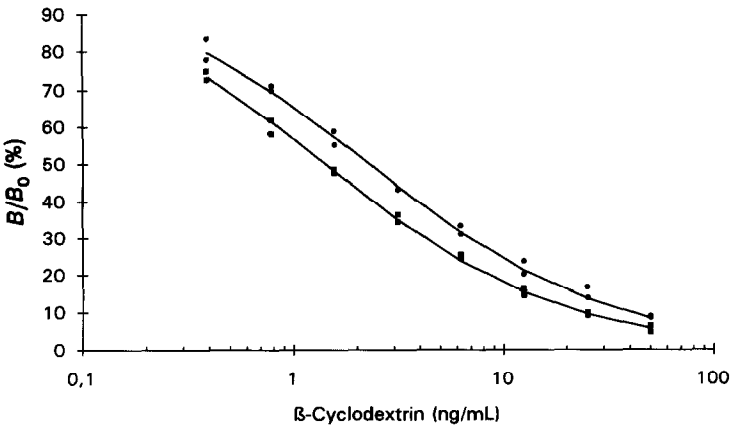


Fig. 2. Enzyme immunoassay titration curves for cyclomaltoheptaose performed in urine (●) and in buffer after extraction from plasma (■).

Cross reactivity in the presence of self-inclusion complexes.—The effect of the state of the cyclodextrin cavity (occupied or vacant) on the recognition by the antibodies was also evaluated. Among all covalent derivatives of **2**, four amino acid derivatives [16] (**8**, **12–14**) and three acylated derivatives [17] (**9–11**) were considered. Among the amino acid derivatives, it has been shown [16] by NMR spectroscopy that the phenylalanyl **12** and tyrosyl **13** derivatives behave as self-inclusion complexes in solution, the aromatic moiety of the amino acid being included in the cavity of its own cyclodextrin carrier. This process of self inclusion does not occur for the glycinyl and tryptophanyl derivatives **8** and **14**, the latter being too bulky to fit into the cavity. All derivatives are, however, recognized to various degrees by the antibodies, indicating that the self inclusion process does not induce important variations of the cyclodextrin structure at the level of the epitope. Excellent recognition patterns are also observed for all acylamido derivatives **9–11**. Although it has been shown [17] that, in the case of long aliphatic chains as in **11**, the hydrocarbon moiety is totally folded into the cavity of the cyclodextrin, these molecules exhibit a good cross-reactivity in the assay, further supporting the location of the epitope at the secondary hydroxyl groups on the rim side.

Enzyme immunoassay of β-cyclodextrin in biological fluids.—Standard determinations of cyclomaltoheptaose were also performed on biological fluids. The potential application of the EIA assay is indeed to quantitatively determine intact cyclomaltoheptaose or its derivatives in biological fluids and tissues after oral or parenteral administration of inclusion complexes for therapeutic purposes. In human urine, it was observed that this EIA determination could be readily performed without loss of sensitivity (Fig. 2). When directly assayed in normal human plasma, the enzyme immunoassy of β-cyclodextrin lacked reproducibility. After extraction with methanol, evaporation of the supernatant and redissolution in EIA buffer, the assay appeared as sensitive and reproducible as when performed in pure buffer as evidenced in Fig. 2.

4. Conclusions

From the present results, a sensitive, specific, and reliable enzyme immunoassay for β-cyclodextrin is now available. It exhibits a good sensitivity and accuracy in different biological media. Recognition of β-cyclodextrin seems to involve the wider secondary rim, since β-cyclodextrin derivatives substituted at OH-2 or -3 failed to be detected. Moreover, all derivatives of cyclomaltoheptaose bearing chemical modifications at primary hydroxyl sites are recognized. Owing to the long life time of β-cyclodextrin in biological media, this assay seems totally suitable for pharmacological studies during the elaboration and validation of pharmaceutical preparations using the important inclusion properties of β-cyclodextrin.

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