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A sensitive and specific enzyme immunoassay for the detection of methyl ether derivatives of cyclomaltoheptaose

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Abstract

We have raised antibodies against two methylated derivatives of β-CD, heptakis(2,6-di-*O*-methyl)cyclomaltoheptaose (Dimeb) and heptakis(2,3,6-tri-*O*-methyl)cyclomaltoheptaose (Trimeb). These antibodies were used to develop two specific and sensitive enzyme immunoassays, presenting a detection limit close to 500 and 30 pg/mL for Trimeb and Dimeb, respectively. Cross reactivities of different linear and cyclic maltooligosaccharides were investigated, demonstrating a high specificity against the structural features of the secondary hydroxyls rim. Several commercial Dimeb samples, containing different mixtures of partially methylated β-cyclodextrin derivatives including RAMEB, which contains only a few amount of pure Dimeb, could be easily evaluated by the Dimeb immunoassay. Both of these assays have been shown to allow accurate measurement in plasma and urine, thus appearing as useful tools for further applications in biological material.

Keywords: Immunoassays; Methylated cyclodextrins; Dimeb; Trimeb; Plasma and urine detection

1. Introduction

Among the natural molecules, cyclomaltooligosaccharides (cyclodextrins, CDs) present the unique feature of entrapping small organic compounds into their hydrophobic cavity. This inclusion process leads to modifications of the properties of the guest molecules, particularly in terms of solubility and stability or modification of the reactivity. Cyclodextrins are thus used in numerous industrial fields, for example in the food industry to stabilize flavors or spices and to avoid oxidation or photoreaction.[1] However, the most promising prospects for these compounds rely on cosmetic or pharmaceutical applications which involves their use for stabilization of the guest active compounds or increasing their bioavailability and eventually reducing the side-effects of drugs.[2,3] Besides demonstrating the pharmaceutical interest of using CD in these formulations, a safety evaluation must be carried out to provide informations to the regulatory agencies before approval of a new CD-based drug formulation.[4] These studies require sensitive and specific methods dedicated to quantitatively measure CD in biological materials. We have recently reported the production of specific polyclonal antibodies against cyclomaltoheptaose (β-CD),[5] cyclomaltooctaose (γ-CD) and derivatives, allowing the development of sensitive immunoassays which proved to be useful for performing a pharmacokinetic analysis for these compounds after intravenous or oral administration to rats.[6,7] A similar strategy is presently used to develop sensitive immunoassays for the methylated derivatives of β-CD, which present interesting potential for various industrial purposes. We have raised antibodies against two methylated derivatives of β-CD, heptakis(2,6-di-*O*-methyl)cyclomaltoheptaose (Dimeb) and heptakis(2,3,6-tri-*O*-methyl)cyclomaltoheptaose (Trimeb).

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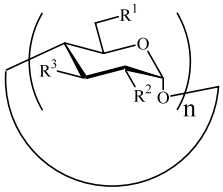
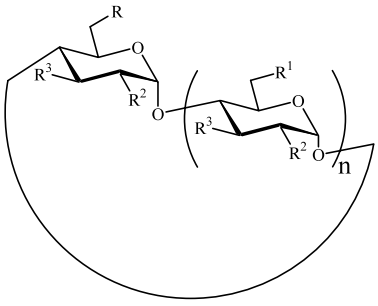




1 n = 6 $R^1 = R^2 = R^3 = OH$

2 n = 7 $R^1 = R^2 = R^3 = OH$

3 n = 8 $R^1 = R^2 = R^3 = OH$

5 n = 7 $R^1 = R^2 = OCH_3$ $R^3 = OH$

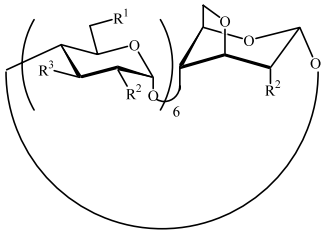
8 n = 7 $R^1 = R^2 = R^3 = OCH_3$

10 n = 8 $R^1 = R^2 = OCH_3$ $R^3 = OH$

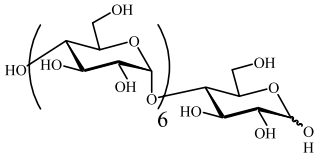
4 n = 6 $R = NH_2$ $R^1 = R^2 = R^3 = OH$

6 n = 6 $R = NH_2$ $R^1 = R^2 = OCH_3$ $R^3 = OH$

9 n = 6 $R = NH_2$ $R^1 = R^2 = R^3 = OCH_3$



7 $R^1 = R^2 = OCH_3$ $R^3 = OH$



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Scheme 1. Linear and cyclic maltooligosaccharides used in the present study.

Cross reactivities of different linear and cyclic maltooligosaccharides were investigated (Scheme 1), demonstrating a high specificity against the structural features of the secondary hydroxyls rim. It will be demonstrated that several commercial Dimeb samples, containing various different mixtures of partially methylated β-cyclodextrin derivatives can be easily detected and evaluated using the Dimeb immunoassay. Both Dimeb and Trimeb immunoassays allow accurate measurements in plasma and urine, thus appearing as useful tools for further applications in biological materials.

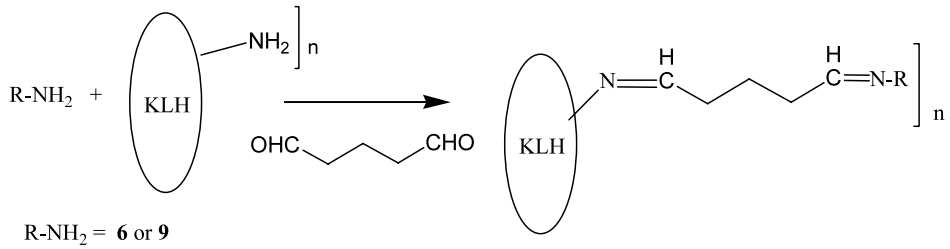
2. Results and discussion

2.1. Development of the immunoassay for Trimeb

As previously described for cyclomaltoheptaose[5] and cyclomaltooctaose,[6] substitution of a single primary alcoholic group by an amine functionality proved to be a very efficient strategy to develop this enzyme immunoassay. We have used the glutaraldehyde method applied to **9** to ensure covalent coupling between the hapten and the carrier molecule, i.e., keyhole limpet hemocyanin (KLH), as described in Scheme 2. The strategy used to obtain the corresponding enzymatic tracer is displayed on Scheme 3.

Indeed, a very good antibody titer was observed once again, as soon as the first bleeding, in the sera of the three immunized rabbits (close to 1/100,000) by performing binding experiments with enzymatic tracer using serial dilutions of the sera. This reflects a strong immunogenic potency of the molecule coupled to KLH and further demonstrates that the addition of the methyl groups all over the hydroxyl functions of the cyclomaltoheptaose molecule maintains a high response of the immune system, as previously observed for the parent β-CD. If the titers of the sera virtually remain unchanged after the next booster injections, the sensitivity improved during the immunization process. Finally, the typical standard curves obtained with the sera (1/100,000 dilution) of the three rabbits are shown in Fig. 1 and displayed very close properties.

The sensitivity at B/B_0 50% ranged between 3.5 and 5.5 ng/mL (123 and 193 fmol/well), since it was calculated at 4.36, 3.56 and 5.42 ng/mL for sera L1146, L1147 and L1148, respectively. The minimum detectable concentration for the best serum (L1147) was close to 500 pg/mL (17.5 fmol/well). The precision of the assay was also very satisfactory as a coefficient of



Scheme 2. Synthesis of immunogens. Reactions conditions: 0.1 M phosphate buffer, 4 °C 18 h. KLH: keyhole limpet hemocyanin.

variation below 11% was observed in the 0.75–100 ng/mL range (data not shown). It is worth noting that the immunization process was in the case of this molecule rather short (limited to 3 booster injections). The extension of the immunization protocol, i.e., further booster injections, could have permitted to reach a better sensitivity of the immunoassay even if the presently obtained ng/mL range of the assay appears not as unsatisfactory.

2.2. Development of the immunoassay for Dimeb

The same approach, i.e., the use of a synthetic monoamino derivative of Dimeb **6** to prepare the immunogen and the enzymatic tracer, was then undertaken. The immunogenic potency characterizing the different cyclodextrins previously studied was once again observed. Among the three immunized rabbits, one died rapidly after the first booster injection (the cause of death was a spinal column breaking, thus unrelated to a possible toxic effect of the immunogen) while the two others exhibited a good antibody titer ($>1/100{,}000$). Moreover, these early antisera also provided standard curves showing a pretty good sensitivity, ranging between 2 and 5 ng/mL. The further immunization process did not allow a strong increase in the titer of the sera. However, after 5 booster injections corresponding to the end of the protocol, the optimized immunoassay developed using the Dimeb-AchE tracer was very sensitive. A typical routine standard curve for an assay using antiserum (L1144S7) at an initial dilution of 1/200,000 is shown in Fig. 2. The sensitivity at B/B_0 50% was about 134 pg/mL (5 fmol/well), with a minimum detectable concentration close to 30 pg/mL (1.1 fmol/well). The assay also exhibited a good precision, as shown by a coefficient of variation below 12% in the 1250-39 pg/mL range.

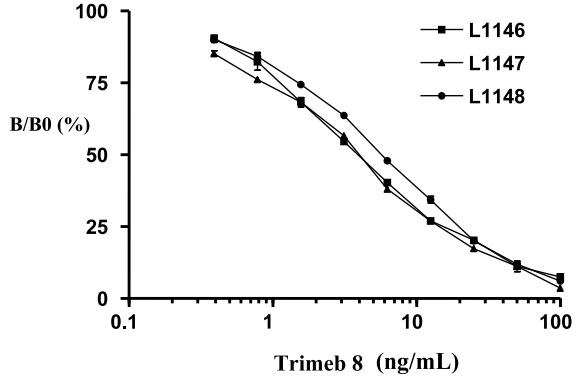


Fig. 1. Standard curve for Trimeb **8** enzyme immunoassay performed in EIA buffer using the antisera from the three immunized rabbits.

2.3. Specificity of the assays for the methyl ether derivatives of cyclomaltoheptaose and cyclomaltooctaose

To characterize the specificity of the Dimeb and Trimeb antibodies, standard curves were performed with various methyl ether derivatives of cyclomaltoheptaose and cyclomaltooctaose. The synthesis of octakis(2,6-di-*O*-methyl)cyclomaltoctaose (**10**) was already described using a protection–deprotection strategy,[8,9] however, characterization of **10** is not fully satisfactory. We describe a direct and efficient synthesis using standard conditions together with a complete characterization of **10**.

The specificity of the polyclonal antibodies raised against heptakis(2,6-di-*O*-methyl)cyclomaltoheptaose (**5**) and heptakis(2,3,6-tri-*O*-methyl)cyclomaltoheptaose (**8**) was characterized by performing standard curves with various derivatives. The results are shown in Table 1 and expressed in terms of percentage cross-reactivity (CR) corresponding to [(dose of Dimeb or Trimeb at B/B_0 50%/dose of analog at B/B_0 50%)] × 100.[10]

For anti-heptakis(2,6-di-*O*-methyl)cyclomaltoheptaose antibodies, the recognition was strictly restricted to **5** or to the amino derivative **6** used to prepare the immunogen and the enzymatic tracer. It is noteworthy that the cross-reactivity obtained with this analog is very close to the parent compound, thus demonstrating that the amino group is not necessary to the binding by the antibodies. Neither the natural cyclic compounds related or not to the molecule, i.e., cyclomaltoheptaose (**2**), cyclomaltohexaose (**1**) and cyclomatooctaose (**3**), nor the linear analog **11** exhibited any cross reactivity. Moreover, heptakis(2,3,6-tri-*O*-methyl)cyclomaltoheptaose **8** or its monoamino derivative **9** and obviously octakis(2,6-di-*O*-methyl)cyclomaltooctaose (**10**) were not recognized too. This result shows that the tridimensional macrocyclic structure of the molecule as well as

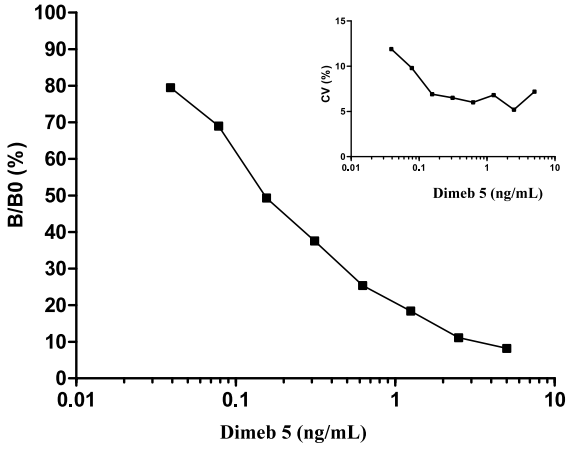
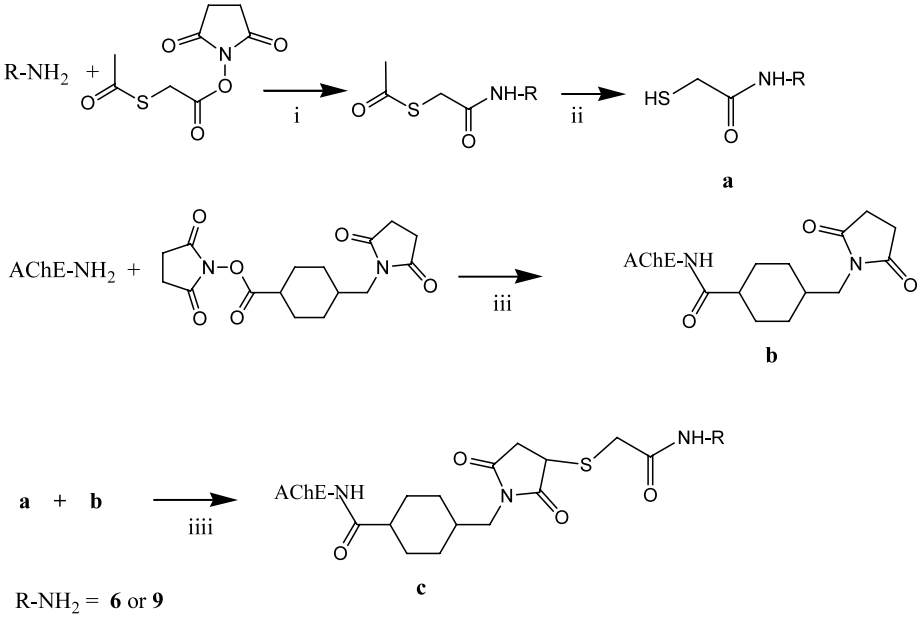


Fig. 2. Standard enzyme immunoassay titration curve for Dimeb **5** performed in EIA buffer. Insert: 'precision profile' for the present assay using data collected for the standard curve with eight replicates for each dose of Dimeb **5**. The precision of the assay is expressed as the coefficient of variation CV vs. the dose (logarithmic scale).

the OH groups at C-3 play a key role for high antibody affinity, hypothetically involving the formation of hydrogen bond(s) inside the immunological complex. These observations are in good agreement with previous results obtained during the course of development of antibodies against cyclomaltoheptaose and cyclomaltooctaose using a similar strategy which have shown the major involvement of the wider secondary rim of the cyclodextrin molecule in the recognition by the antibodies. Finally, only a monoanhydro derivative of Dimeb **7** displayed a good cross reactivity, revealing that the internal link between C-3 and C-6 does not generate dramatically strong structural modifications of the backbone of the molecule. These results are in agreement with previous data,[11] where it has been demonstrated that the formation of one single 3,6-anhydro bridge destroyed the sevenfold symmetry of the β-cyclodextrin without affecting the overall dimensions of the molecule.

On the other hand, this new and specific immunoassay for heptakis(2,6-di-*O*-methyl)cyclomaltoheptaose was used to analyze Dimeb derivatives **12**–**17** available from different sources, i.e., some commercially available and others resulting from industrial collaboration, since the synthesis and production of chemically pure Dimeb is difficult to achieve. NMR and mass spectrometry experiments were performed to estimate the purity of the different batches. To summarize the physico-chemical data, **12**–**17** are mixtures of roughly overmethylated and submethylated derivatives of heptakis(2,6-di-*O*-methyl)cyclomaltoheptaose as shown in Table 2. Compound **5** contains more than 99% of the correct product and was then used as standard for all Dimeb immunoassay experiments. Among the 6 other Dimeb derivatives tested, the quality appears quite variable. Three batches, **12**–**14** corresponding to different steps of purification as obtained from the industrial partner, show less than 11% of contamination by other CDs, identified as 3 or 4 overmethylated isomers. For the commercial products **15** and **16**, the extent of contamination largely increases, exceeding 50% and including a large number of overmethylated analogues of heptakis(di-2,6-*O*-methyl)cyclomaltoheptaose. When tested for immunoreactivity using the Dimeb immunoassay, the five products proved to be fully cross reactive.



Scheme 3. Synthesis of enzymatic tracers. Reactions conditions: (i) 49:1 0.1 M phosphate buffer: DMF, RT, 30 min; (ii) NH_2OH, 0.1 M phosphate buffer, RT, 30 min; (iii) 0.1 M phosphate buffer, RT; 15 min; (iv) 0.1 M phosphate buffer, 30 °C, 3 h.

Table 1
Relative cross-reactivity (CR) obtained with compounds **1**–**11** using anti-Dimeb and anti-Trimeb antibodies.

Compound	CR (%) (Dimeb assay)	CR (%) (Trimeb assay)
Cyclomaltohexaose (**1**)	<0.1	<0.1
Cyclomaltoheptaose (**2**)	<0.1	<0.1
Cyclomaltooctaose (**3**)	<0.1	<0.1
6^{I}-Amino-6^{I}-deoxy-cyclomaltoheptaose (**4**)	<0.1	<0.1
Heptakis(2,6-di-*O*-methyl)cyclomaltoheptaose (**5**)	100	0.8
6^{I}-Amino-6^{I}-deoxy-2^{I}-*O*-methyl-hexakis(2^{II-VII},6^{II-VII}-di-*O*-methyl)cyclomaltoheptaose (**6**)	103	0.8
3^{I},6^{I}-Anhydro-2^{I}-*O*-methyl-hexakis-(2^{II-VII},6^{II-VII}-di-*O*-methyl)cyclomaltoheptaose (**7**)	53	1.4
Heptakis(2,3,6-tri-*O*-methyl)cyclomaltoheptaose (**8**)	<0.1	100
6^{I}-Amino-6^{I}-deoxy-2^{I},3^{I}-di-*O*-methyl-hexakis(2^{II-VII},3^{II-VII},6^{II-VII}-tri-*O*-methyl)cyclomaltoheptaose (**9**)	<0.1	98
Octakis(2,6-di-*O*-methyl)cyclomaltoctaose (**10**)	<0.1	<0.1
Maltoheptaose (**11**)	<0.1	<0.1

Finally, Rameb **17** was also tested using the two different immunoassays. This commercial product is in fact a complex heterogeneous mixture, as shown by MS analysis[12,13] (Fig. 3).

Electrospray ionization of **17** with positive ion detection leads to a series of protonated molecules , $[M+H]^+$, at m/z values depending on the number of methyl group in each individual sugar unit of the cyclodextrin derivative. The mass spectra reflect the average distribution of the methyl groups of **17**. It should be noted that the ion peak at m/z 1331 corresponding to 14 *O*-methyl groups (i.e., heptakis(2,6-di-*O*-methyl)cyclomaltoheptaose in first approximation) represents only 9% of the total amount. It should be pointed out that the other methylated derivatives present in Rameb exhibit a lower degree of methylation (between 9 and 13 *O*-methyl groups) while Trimeb is not detected. Compound **17** is then a mixture of submethylated derivatives of heptakis(2,6-di-*O*-methyl)cyclomaltoheptaose. The Dimeb assay exhibited a lower but significant cross reactivity (11.5%) by comparison with the other studied compounds **12**–**16**.

This result shows that the contaminant CDs (overmethylated at C-3 or submethylated at C-2), i.e., different from true Dimeb, present in the samples are recognized. The above specificity studies and previously published results have shown that the C-6 positions are not clearly involved in the recognition by the antibodies and thus their modification does not affect the cross reactivity. Conversely, the secondary hydroxyl rim mainly corresponds to the recognized epitope. However, the results obtained with these different samples demonstrated that modification of one or two glucopyranose unit(s) at C-2 and/or C-3 is tolerated for the binding by the antibodies, in accordance with the results obtained with the monoanhydro derivative **7**.

For anti-heptakis(2,3,6-tri-*O*-methyl)cyclomaltoheptaose antibodies, the pattern of recognition appears roughly similar. The natural cyclic **1**–**3** or linear **11** compounds were not recognized by the antibodies. Heptakis(2,3,6-tri-*O*-methyl)cyclomaltoheptaose (**8**) and its monoamino derivative **9** were fully recognized, while octakis(2,6-di-*O*-methyl)cyclomaltooctaose (**10**) failed to present any cross-reactivity. However, the

Table 2
Relative cross-reactivity (CR) obtained with Dimeb from different sources using anti-Dimeb and anti-Trimeb antibodies

Compounds	Dimeb (%)	Other CDs (%)	Number of isomers	CR (%) Dimeb assay	CR (%) Trimeb assay
5	99.01	0.9	1	100	0.8
12	94.33	5.66	>2	101.2	0.9
13	92.6	7.40	>2	103.1	0.8
14	90.1	9.90	>3	98.2	0.9
15	44.6	55.7	n	108.3	1.9
16	27.14	72.8	n	96.1	2.5
17	9	91	n	11.5	0.1

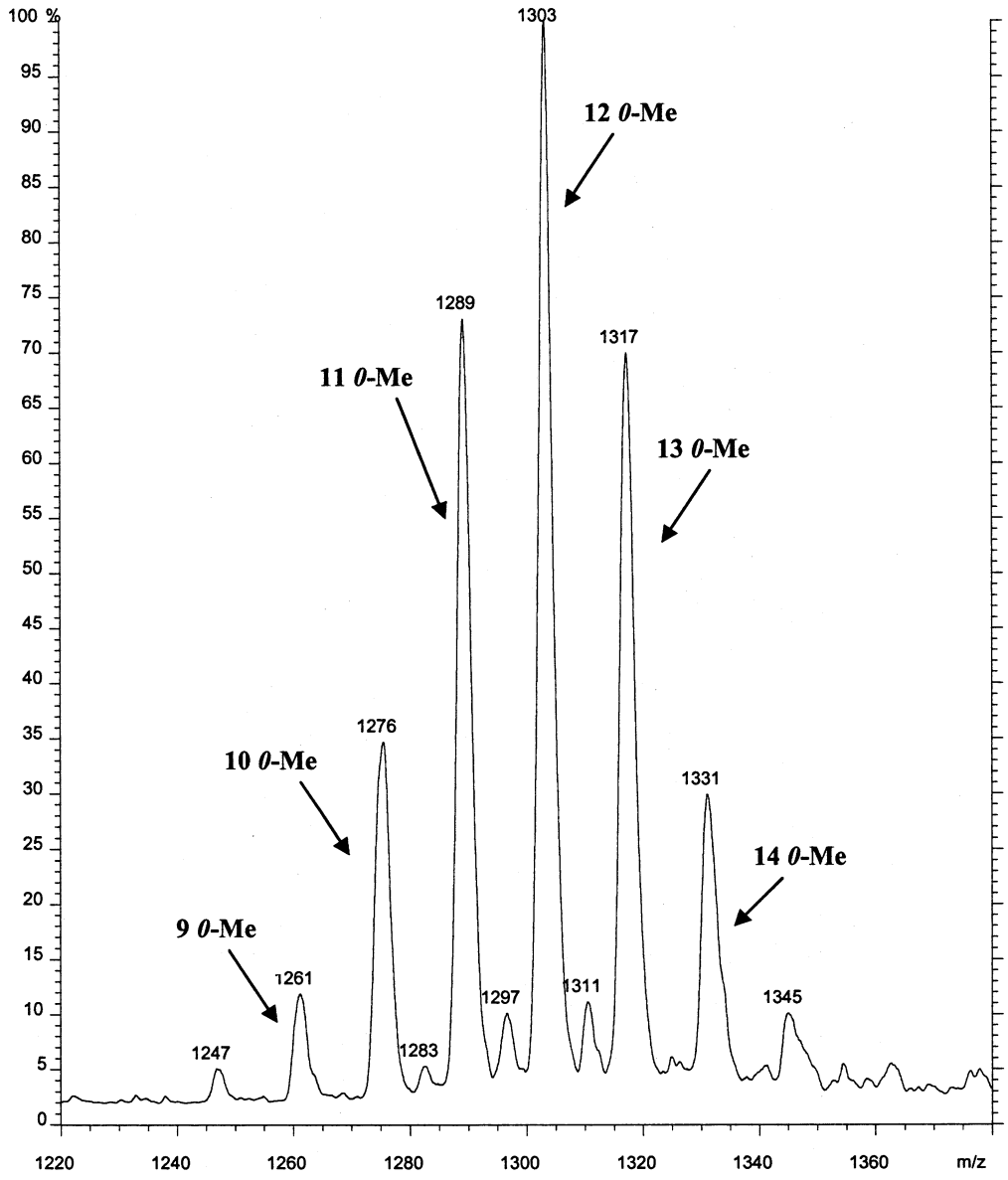


Fig. 3. ESI mass spectra of Rameb **17**, $[M+H]^+$ ions area. (infusion at 5 μL min^{-1} of a 0.01 mg mL^{-1} solution in 1:1 water–MeCN containing 0.1 % TFA) with positive ion detection.

Dimeb related compounds, i.e., **5** and **12**–**16** exhibited a close low but significant cross reactivity, ranging between 0.8 and 2.5%. In fact, the cross reactivity increases with the amount of overmethylated derivatives and more specifically with the percentage of additional methylation at C-3. These results show that methylation at C-3 favors the recognition but its undermethylation does not totally suppress binding by the antibodies. Moreover, the low recognition of the monoanhydro derivative strengthens the above conclusion concerning the keeping of a global conformation despite this internal bond. Rameb **17** exhibited no significant cross reactivity using the anti-Trimeb antibodies, confirming the above analytical results.

2.4. Enzyme immunoassay of Dimeb in biological fluids

The above experiments show that the present Dimeb immunoassay could be used to investigate the fate of Dimeb from different sources and quality and even Rameb **17** during in vitro or in vivo experiments. However to evaluate the potential application of this immunoassay to quantitatively measure intact Dimeb in biological fluids and tissue extracts after administration of inclusion complexes for therapeutic purposes, standard determinations of Dimeb were also performed in biological fluids (urine and plasma).

Antiserum dilution experiments performed in plasma show that the antiserum titer was only slightly modified

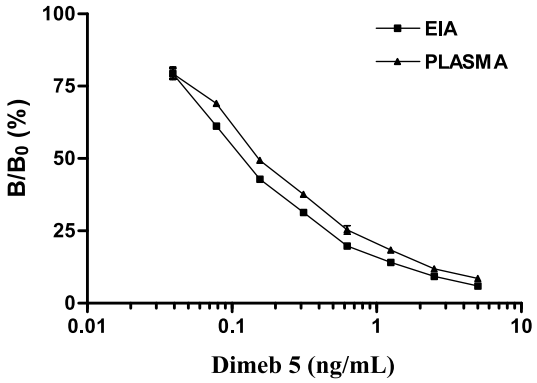


Fig. 4. Standard curves for Dimeb **5** in EIA buffer (■) or in plasma (▲) after 1 week incubation at +4 °C as detected with the specific enzyme immunoassay.

(1/100,000 by comparison to 1/200,000 in EIA buffer). Normal plasma or urine were spiked with known amounts of **5** and assayed either immediately or, for plasma, after one week storage at +4 °C. The results were compared to a standard curve performed in EIA buffer in the same conditions.

As shown in Fig. 4, the results obtained after one week incubation in EIA buffer (B/B_0 50% = 124 pg/mL) or plasma (B/B_0 50% = 177 pg/mL) are quite similar and presented no difference with the initial measurements (B/B_0 50% = 140 and 136 pg/mL in EIA buffer and plasma, respectively; data not shown). This experiment demonstrates the stability of this methylated CD derivative in plasma under these storage conditions. Performing the assay in urine requires, as observed for plasma, to slightly modify the antiserum dilution (close to 1/100,000). Standard curve showed that the sensitivity of the assay was essentially preserved in this medium (B/B_0 50% = 280 pg/mL; data not shown). We have not checked the conservation of Dimeb **5** in urine as previously shown for plasma.

These results demonstrates that this immunoassay could be performed directly in such biological fluids. However, it is possible that some important interferences will be encountered when assaying numerous plasma or urine samples. Moreover, the pharmacokinetic analysis of cyclodextrins would imply to assay samples presenting concentrations in a large range, i.e., from μg/mL to pg/mL as previously reported.[6,7] The quantitative measurement of these samples will thus require, prior using immunoassay, a dilution process and to analyze very different dilutions to correctly fit the standard curve. Since we have observed a small interference of these biological fluids on the binding of the tracer to the antibodies, as revealed by the modification of the antiserum titer, this dilution process if performed in buffer could lead to over- or under-evaluation of Dimeb **5** content in the diluted samples. To overcome these different problems, it could appear judicious to apply systematically to these samples a simple methanol extraction protocol (see Section 4) which has previously proved to be useful.[6,7] After evaporation of the supernatant, samples were reconstituted with EIA buffer, thus ensuring a good sensitivity and reproducibility of the assay. When tested by spiking control plasma and urine samples with known amounts of Dimeb **5**, this extraction method demonstrated more than 95% of recovery of the molecule in the supernatant.

3. Conclusions

The present results show that two different reliable and specific enzyme immunoassays are now available for two methyl derivatives of β-cyclodextrin, namely heptakis(2,6-di-*O*-methyl)cyclomaltoheptaose (Dimeb) and heptakis(2,3,6-tri-*O*-methyl)cyclomaltoheptaose (Trimeb). These assays exhibit good sensitivity and could be accurately applied for measurement in different biological media. The strategy used to raise the antibodies, e.g., the use of a mono-amino derivative at carbon C-6, directed the specificity of the antibodies against the secondary hydroxyls rim of the molecules. The recognition of Trimeb by the antibodies involves the methoxy groups at C-2 and C-3 on at least 5 or 6 consecutive maltose units of the core of β-CD. The specificity of anti-Dimeb antibodies relies on the succession of *O*–Me and OH groups at C-2 and C-3, respectively, tolerating the absence of methoxy group at C-2 or its introduction at C-3 in a limited way as suggested by the full recognition of some isomers or the limited cross reactivity of Rameb during specificity analysis experiments.

4. Experimental

4.1. Reagents and buffer

Unless otherwise stated, all reagents were of analytical grade, from Sigma (St. Louis, USA). β-CD, α-CD and γ-CD were gifts from Roquette Frères (Lestrem, France) and Wacker (Germany). Heptakis(2,3,6-tri-*O*-methyl)cyclomaltoheptaose (**8**) and maltoheptaose (**11**) were purchased from Fluka AG (Switzerland) and used without further purification. Compounds **5** and **12**–**17** are commercial heptakis(di-2,6-*O*-methyl)cyclomaltoheptaose derivatives containing different mixtures of partially methylated β-cyclodextrin derivatives. The number of compounds in the dedicated mixture and the overall degree of methylation have been checked by digital integration of the ^{1}H NMR spectra and by mass spectrometry. The main results are summarized in Table 2. Compounds **5**, **12**, **13** and **14** were kindly donated by Cis bio international (Saclay, France) and were obtained

in one step from the parent β-cyclodextrin as described elsewhere.[14] The degree of purity depends on the number of recristallizations of the crude material in hot water. Compound **5** will be considered as pure heptakis(di-2,6-*O*-methyl)cyclomaltoheptaose and used as reference. Compounds **15**–**17** were purchased from Fluka, Acros and Wacker respectively and used without any purification. 6^{I}-Amino-6^{I}-deoxy-cyclomaltoheptaose (**4**), 6^{I}-amino-6^{I}-deoxy-2^{I}-*O*-methyl-hexakis(2^{II-VII},6^{II-VII}-di-*O*-methyl)cyclomaltoheptaose (**6**), 3^{I},6^{I}-anhydro-2^{I}-*O*-methyl-hexakis(2^{II-VII},6^{II-VII}-di-*O*-methyl)cyclomaltoheptaose (**7**) and 6^{I}-amino-6^{I}-deoxy-2^{I},3^{I}-di-*O*-methyl-hexakis(2^{II-VII},3^{II-VII},6^{II-VII}-tri-*O*-methyl)cyclomaltoheptaose (**9**) were prepared as already described.[15,16] The chemical and optical purity of the different compounds was checked by ^{1}H NMR, mass spectroscopy and chemical analysis and compared to literature data.

4.2. Octakis(2,6-di-*O*-methyl)cyclomaltoctaose (10)

Barium oxide (3 g, 20 mmol) and barium hydroxide octahydrate (3 g, 9.5 mmol) were successively added to a solution of γ-CD (2 g, 1.5 mmol) in 1:1 dry Me_2SO–DMF. The mixture was stirred under nitrogen at 8–9 °C for 3 days. Concentrated ammonium hydroxide (5 mL) was then added and the mixture was stirred for a further 3 h at room temperature. After evaporation of most of the solvents, the resulting solid cake was extracted with CH_2Cl_2 (3×20 mL). The combined fractions were washed with saturated aq sodium chloride solution (3×20 mL) and water (20 mL), dried over sodium sulfate and concentrated under diminished pressure. The crude product was precipitated by addition of *n*-hexane (100 mL), filtered, washed with *n*-hexane and dried. The final product (1.79 g, 74%) was obtained by 2 successive recrystallizations in boiling water; mp 160–164 °C (dec), $[\alpha]_D^{24}$ +129° (*c* 1.2, $CHCl_3$), lit[9]: $[\alpha]_D^{24}$ +127° (*c* 1, $CHCl_3$); ^{1}H NMR (500 MHz, D_2O, 298 K): δ 5.11 (d, 7 H, $J_{1,2}$ 2.5 Hz, H-1), 3.86 (t, 7 H, $J_{2,3} = J_{3,4}$ 10.0 Hz, H-3), 3.78 (ddd, 7 H, $J_{4,5}$ 10.0 Hz, $J_{5,6}$ 5.1 Hz $J_{5,6'}$ 2.15 Hz, H-5), 3.62 (m, 14 H, H-6 H-6′); 3.47 (t, 7 H, $J_{3,4} = J_{4,5}$ 10.0 Hz, H-4); 3.44 (s, 21 H, CH_3 at C-6); 3.27 (s, 21 H, CH_3 at C-2), 3.25 (dd, 7 H, $J_{1,2}$ 2.5 Hz, $J_{2,3}$ 10.0 Hz, H-2); ES-HRMS: *m*/*z* 1543.6633 $[M+Na]^+$, $C_{64}O_{40}H_{112}Na$ requires 1543.6627.

Acetylcholinesterase (AChE, EC 3.1.1.7) from the electric organs of the electric eel *Electrophorus electricus* was purified by affinity chromatography[17] and used in its G4 form for labelling.[18] AChE activity was measured using the colorimetric method of Ellman et al.

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. The washing buffer was a 10 mM phosphate buffer pH 7.4 containing 0.05% Tween 20.

4.3. Preparation of the immunogens and antiserum production

For the production of antibodies, the amino derivatives were covalently linked to keyhole limpet hemocyanin (KLH) using glutaraldehyde (Scheme 2). Briefly, 7 μmol of amino-CD, corresponding to 9.31 mg of **6** or 9.89 mg of **9**, respectively, were mixed with 20 mg of KLH in 10 mL of 0.1 M phosphate buffer pH 7.4 before adding 40 μL of glutaraldehyde (25% in water). After stirring 18 h at +4 °C in the dark, the immunogens were dialyzed against 0.1 M phosphate buffer pH 7.4 to remove the unreacted glutaraldehyde, aliquoted without any further treatment and stored frozen at −20 °C until use.

Rabbits (Blanc du Bouscat, Evic, France) were immunized with 1 mg of immunogen using complete Freund's adjuvant and multiple subcutaneous injections. Booster injections (1 mg of immunogen in complete Freund's adjuvant) were given every month for 6 months. Rabbits were bled from the central ear artery one week after each booster injection. Blood was centrifuged, and sera were stored at 4 °C in the presence of sodium azide (0.01% final concentration).

4.4. Preparation of the enzymatic tracer

The amino derivatives were covalently coupled to AChE using the heterobifunctional reagent *N*-succinimidyl-4-(*N*-maleimidomethyl)-cyclohexane-1-carboxylate (SMCC) as previously described.[19] This method involved the reaction of thiol groups previously introduced in **6** or **9**, by reaction of its primary amino group with *N*-succinimidyl-*S*-acetylthioacetate (SATA) in alkaline medium with maleimido groups incorporated into the enzyme as described in Scheme 3. The enzymatic tracer was purified by filtration onto sepharose gel.

4.5. Competitive EIA procedure

Solid phase competitive EIA was performed on 96-well microtiter plates (Immunoplate Maxisorb with certificate, Nunc, Denmark) using an automatic Titertek microtitration equipment (washer, dispenser and reader) from Labsystems (Helsinki, Finland), as previously described.[5,7,19,20] Microtiter plates were coated with mouse monoclonal *anti*-rabbit IgG antibodies in order to ensure separation between bound and free moieties of the enzymatic tracer during the immunological reaction. The coating procedure (200 μL/well of a 5 μg/mL monoclonal antibody solution in 0.05 M phosphate buffer, pH 7.4) was performed for 18 h at room temperature, before washing the plates (300 μL/well

and three wash cycles) and saturating for 24 h at 4 °C with EIA buffer (300 μL/well). Coated plates covered with an adhesive plastic sheet were stable for at least 3 months when stored at +4 °C. After washing the coated microtitre plate, the assay was performed in a total volume of 150 μL. To each well were successively added: 50 μL of calibrator, buffer or sample, 50 μL of enzyme tracer and 50 μL of diluted antiserum. The enzymatic tracer was used at a concentration of 2 Ellman units/mL (for Ellman unit definition, see Ref. [20]). The working dilution for the rabbit antiserum (ranging generally from 1/10,000 to 1/1000,000) was determined by performing serial dilution experiments.

After a 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 Ellman's reagent[21] (corresponding to a solution of 7.5×10^{-4} M acetylthiocholine iodide, enzyme substrate, and 5×10^{-4} M 5,5′-dithiobis-(2-nitrobenzoic acid), chromogen in 0.1 M phosphate buffer pH 7.4) in each well. After 1 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 in the absence of competitor, respectively. A linear log–lin transformation was used to fit the standard curve.[22] The sensitivity of the assay was characterized by the dose of competitor 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 determinations were made in duplicate and quadruplicate for B_0.

Before assaying plasma and urine samples, a methanol precipitation procedure can be performed.[5] Four vol of cold MeOH were added to the sample before centrifugation (2500 rpm, 10 min). The pellet was discarded and the supernatant dried under diminished pressure using a SpeedVac apparatus (Savant, Farmingdale, NY) before reconstitution with EIA buffer.

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