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A Use of Modified Cyclodextrins as a Transporter for a Radiolabeled Tracer NMR Investigation

Y. MICHEL[2], B. PERLY[1], F. DJEDAINI-PILARD[3], E. BERTHOMMIER[2], C. BAUDIN[1], C. FAJOLLES[1], B. HAMANN[2], R. PASQUALINI[2] and L. MAUCLAIRE[1]*
[1]*SCM/DRECAM/DSM, CEA/SACLAY, bât. 125, F-91191 Gif – sur – Yvette Cedex, France;* [2]*Schering/CIS bio-international, B.P. 32, F-91192 Gif – sur – Yvette Cedex, France;* [3]*UPJV, Laboratoire des Glucides, route de St Leu, F-80000 Amiens, France*

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Abstract

Cyclodextrins (D. Duchêne (ed.): *New Trends in Cyclodextrins and Derivatives* (1991)) have long been shown to be capable of modifying the water solubility of a number of hydrophobic guests through the formation of inclusion complexes. Among the three natural cyclodextrins (α, β and γ-cyclodextrins containing 6, 7 and 8 D-glucopyranose units, respectively), β-cyclodextrin is by far the most commonly used although it exhibits a weaker solubility in water (and therefore a weaker solubilization power). This specific feature has encouraged the synthesis of derivatives exhibiting an increased solubility in water. Methylated cyclodextrins are amongst the simplest derivatives, and their properties regarding the solubility and the solubilization power for hydrophobic guests are well documented especially concerning Heptakis (2,6-di-Omethyl)-cyclodextrin (DIMEB) and Heptakis (2,3,6-tri-Omethyl)-cyclodextrin (TRIMEB) K. Koizumi *et al.*: *J. Chromatogr.* **368**, 329–337 (1986). In order to avoid the use of human serum albumin (HSA), this property has been applied here to the solubilization of a very sparingly water-soluble fatty acid derivative (16-iodo-3-methylhexadecanoic acid), which is known to localise in viable myocardial cells, allowing the generation of functional images reflecting the viability of the cardiac tissue through the use of radiolabeled analog (Demaison *et al.*: *J. Nucl-Med.* **29**, 1230–1236 (1998)). Nuclear magnetic resonance (NMR) was used throughout this study to evidence that the observed solubilization and stabilisation (under conditions required for sterilisation) induced by cyclodextrins are due to the formation of a true inclusion complex and not to non-specific interactions; This technique further allows to derive thermodynamic as well as structural informations for this complex. On one hand, the inclusion complex prevents thermal degradation during sterilisation process compared to HSA. On the other hand, NMR displacement experiments against HSA showed that the complex likely dissociates *in vivo*.

Introduction

Radiolabeled tracers are of considerable importance in the clinical investigation of organic dysfunctions. Among them, labeled fatty acid have shown excellent specificity in distinguishing between viable and non-viable myocardial tissue and are currently used for diagnostic purposes. Owing to their very low solubility in aqueous media, they have to be injected as solubilized forms. Up to now, the most classical procedure was to use human serum albumin (HSA) as the solubilizing agent, this protein being the natural transporter for fatty acids in blood. Sanitary regulations, however, preclude the use of non-sterilised or potentially contaminated blood components for administration to humans and have strongly stimulated the use of other potential transporters for these compounds.

Cyclodextrins have long been shown to be capable of modifying the water solubility of a number of hydrophobic guests through the formation of inclusion complexes. Among the three natural cyclodextrins (α,β and γ-cyclodextrin containing 6, 7 and 8 D-glucopyranose units, respectively), β-cyclodextrin is by far the most commonly used although it exhibits the weakest solubility in water (and therefore a weaker solubilization power). This specific feature has encouraged the synthesis of derivatives exhibiting an increased solubility in water. Methylated cyclodextrins are among the simplest derivatives and their properties regarding the solubility and solubilization power for hydrophobic guests are well documented especially concerning Heptakis (2,6-di-Omethyl)-cyclodextrin (DIMEB) and Heptakis (2,3,6-tri-Omethyl)-cyclodextrin (TRIMEB). This

* Author for correspondence. E-mail: mauclaire@drecam.cea.fr

property has been applied to the solubilization of a very sparingly water-soluble fatty acid derivative (16-iodo-3-methylhexadecanoic acid), which is known to localise in viable myocardiac tissue, allowing the generation of functional images reflecting the viability of the myocardiac tissue through the use of radiolabeled analog.

We here report the use of a modified cyclodextrin for the solubilization, protection and injection of a labeled fatty acid, namely 16-iodo-3-methylhexadecanoic acid. In clinical investigation of myocardiac dysfunctions, this marker is injected in the form of a (^{123}I)-labeled analog [1]. A detailed investigation using nuclear magnetic resonance (NMR) was carried out to help in selecting the best suited cyclodextrin derivative among seven, to evidence and characterise the formation of an inclusion complex and to get a deeper insight in the capture of the included marker by endogenous HSA. Finally, the benefit of this technique was proven by *in vivo* investigations of the localisation of the marker.

Material and methods

General

α, β and γ cyclodextrins were a gift from Cerestar (Cerestar, Inc, USA) Deuterated water and DMSO-d_6 was supplied by Eurisotop. Eras Labo (Saint Nazaire les Eymes, France) supplied the DIMEB. The other cyclodextrin derivatives and chemicals were purchased from Aldrich (St. Quentin Fallavier, France) and used without further purification.

^{1}H NMR experiments were performed using a BRUKER AMX500 spectrometer operating at 500.13 MHz for proton.

Radio-TLC chromatograms were analyzed on a Berthold TLC-linear analyser (Berthold System, Inc., Pittsburgh, PA USA)

Preparation of inclusion complex samples

The potential for cyclodextrins to improve the solubility in water of (^{127}I) iodo-3-methylhexadecanoic acid was estimated using a preliminary assay. To a 10 mM aqueous solution of the pertinent cyclodextrin derivatives at 25 °C, a 10 mmol dm^{-3} of 16-iodo-3-methylhexadecanoic acid in acetone was added drop wise. The mixture was stirred and the acetone was allowed to evaporate slowly under a stream of nitrogen. Additions were repeated up to saturation characterised by the formation of a turbid solution.

Radiolabeling

About 0.8 mg of (^{127}I) iodo-3-methylhexadecanoic acid is dissolved in 1 ml acetone. About 202 MBq (60 μl) of Na(^{123}I) solution were added and the vial is crimped. This preparation is heated at 105 °C for 5 min, a needle vent allowing the acetone and water to evaporate. When the solution is dry, we made the (^{123}I) iodo-3-methylhexadecanoic acid soluble with a solution of 13 mg of DIMEB in 1 ml of isotonic solution.

The radiochemical purity was determined by thin layer chromatography (TLC) using silica plates developed in *n*-hexane: chloroform (9:1 v:v). R_f (fatty acid) 0.9; R_f (iodide) 0.1

Results and discussion

NMR evidence for the formation of an inclusion complex

The solubility of the 16-iodo-3-methylhexadecanoic acid in water at 25 °C has been determined for all seven cyclodextrin derivatives. The results are displayed in Table 1. From the data presented, three major conclusions can be drawn. The fatty acid is not included in α-cyclodextrin or in TRIMEB. In the case of the hydroxypropyl cyclodextrins, the fatty acid is solubilized but the complexes cannot be studied by NMR since these derivatives are randomly substituted, leading to a very large number of molecular structures. The best results were obtained with β-cyclodextrin derivatives particularly with DIMEB.

NMR spectra of the carrier cyclodextrin derivative were collected in the absence and in the presence of variable amounts of the iodinated fatty acid. Figure 1 shows the results obtained (using deuterium oxide as solvent). Since DIMEB is used as host molecules, large variations of the chemical shifts of protons of the cyclodextrin (mainly H-3 and H-5 located in the hydrophobic cavity) are observed in agreement with the formation of an inclusion complex [2]. From a numerical analysis of the variations of chemical shifts of the CD protons upon controlled addition of the fatty acid and using a least square fitting algorithm, the association constant was found to be 500 ± 50 mol l^{-1} at 25 °C [3]. The reality of the formation of an inclusion complex was further evidenced by the use of a ROESY experiment (Rotating frame OVERHAUSER Effect Spectroscopy) [4, 5] showing dipolar interactions (and therefore spatial proximities) between the fatty acid and the internal cavity of the cyclodextrin. Figure 2 displays a contour

Table 1. Solubility of 16-iodo-3-methylhexadecanoic acid in water and in aqueous solutions of various cyclodextrins

Solvent system	Solubility (mM) at 298 K
Pure water	<0.01
α-CD 10 mM in water	< 0.05
β-CD 10 mM in water	0.8
γ-CD 10 mM in water	2
DIMEB 10 mM in water	5
TRIMEB 10 mM in water	1.5
Hydroxypropyl β-CD 10 mM in water	5
Hydroxypropyl γ-CD 10 mM in water	<5

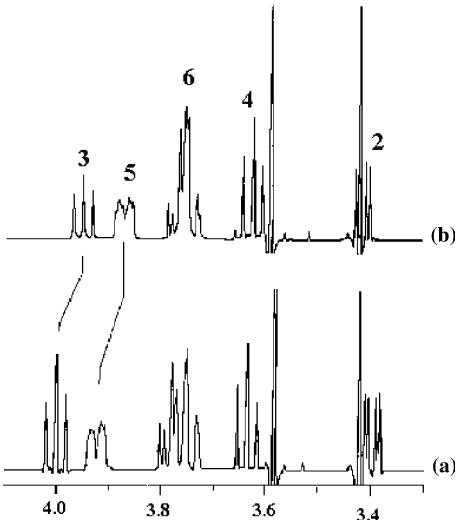

Figure 1. Partial 500 MHz ^{1}H NMR spectra of 3 mM DIMEB alone (a) and in the presence of 1.25 mM (16-iodo-3-methylhexadecanoic acid) (b). Spectra were obtained at 298 K in deuterium oxide containing 100 mM sodium phosphate buffer, pH 7.4 (meter reading) For clarity, the intense singlets from the methoxy groups have been clipped.

plot of a typical experiment with projections; the diagonal represents the normal spectrum and all non-diagonal peaks (cross-peaks) are indicative of spatial proximities between protons (inter-nuclear distance <4 Å). The upper left and lower right corners of the contour plot indeed evidence proximities between signals arising from the fatty acid (2–0.8 ppm) and from the cyclodextrin (4–3.2 ppm) clearly assessing the formation of an inclusion complex.

Sterilisation study of the formulations

The radiopharmaceutical preparation is intended for an intravenous injection. Because the osmotic pressure of the aqueous solution is too low to be safely acceptable, we have looked for an adjuvant, which would be able to increase the osmotic pressure up to physiological values without disturbing the inclusion complex between the iodinated fatty acid and the cyclodextrin. The effect of various pharmaceutical adjuvants on the radiochemical stability of the iodinated fatty acid was tested over different sterilisation cycles. The results are displayed in Table 2. The presence of anionic species in the formulation contributes to accelerate the degradation of the iodinated compound. The best results were obtained with formulations containing 40 mg ml^{-1} of either mannitol or inositol. These preparations display an osmotic pressure between 280 and 300 mOsm kg^{-1}. We have selected mannitol because this compound is described in the European Pharmacopoeia.

A complete investigation (using NMR as an analytical tool) has shown that this adjuvant does not affect (by competition) the formation of the inclusion complex between the modified cyclodextrin and the labeled fatty acid.

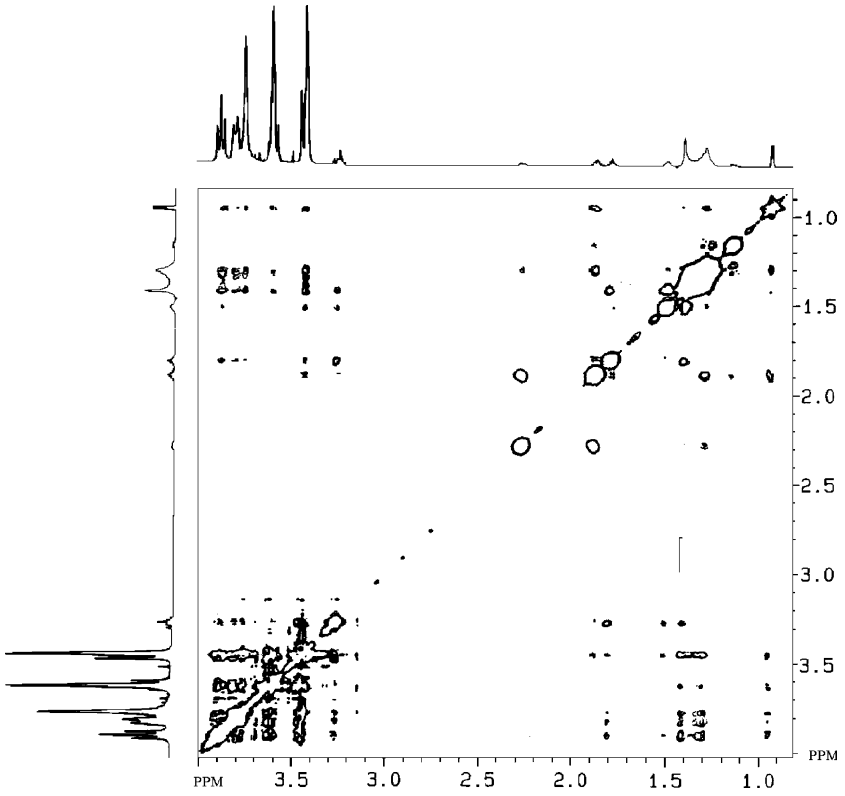

Figure 2. ROESY contour plot (obtained at 500 MHz and 298 K on sample b of Figure 1). Cross peaks show dipolar interactions between host and guest.

Table 2. Effect of the sterilisation conditions on the radiolabeling yields

Cyclodextrin derivative	Sterilisation conditions	Adjuvant	Radiochemical purity (%)	
			Before sterilisation	After sterilisation
2-hydroxypropyl β-CD	110 °C – 30 min	2 ml aqueous buffer CO_3^{2-}/HCO_3^- pH 9	95.5 ± 1.7	78.8 ± 1.3
2-hydroxypropyl β-CD	110 °C – 30 min	2 ml aqueous phosphate buffer pH 7.4	97.2 ± 1.7	91.3 ± 1.0
DIMEB	110 °C – 30 min	2 ml aqueous buffer CO_3^{2-}/HCO_3^- pH 9	95.6 ± 1.6	89.7 ± 0.7
	120 °C – 21 min	H_2O	99.3 ± 1.0	97.2 ± 0.8
	120 °C – 21 min	Inositol solution to 4%	95.8 ± 1.4	90.5 ± 1.2
	120 °C – 21 min	Mannitol solution to 4%	97.8 ± 1.2	96.1 ± 1.0

[(mean ± SD) Test number $n = 5$].

Images of the thoracic region by dynamic acquisitions with a gamma camera were obtained after injection in anaesthetised dogs of the complex of radioiodinated fatty acid in DIMEB. These images were qualitatively compared to those obtained after injection of iodinated fatty acid carried by HSA. The cyclodextrin containing formulation provides contrasted heart images together with a low liver background. These results point out the question of the chemical nature of the material really interacting with the cardiac tissue. It is worth understanding whether, after injection in blood, the labeled fatty acid is bound to the endogenous albumin as a free molecule or as an inclusion complex together with the cyclodextrin carrier.

NMR observation of the capture of the fatty acid by endogeneous human serum albumin (HSA)

In order to address the former point, NMR experiments were carried out to determine whether endogenous albumin carrier was able to remove out the included fatty acid from the complex. Adding human serum albumin (HSA) to a performed inclusion complex results in a progressive transfer of the fatty acid from the cyclodextrin to the protein as shown in Figure 3.

Progressive addition of HSA to the preformed inclusion complex results in a variation of the chemical shifts of protons H-3 and H-5, and, at ca. 200 μM HSA, a typical spectrum corresponding to an empty DIMEB is obtained. The identity between the two spectra displayed in Figure 3d and 3e is quite obvious and further indicates that HSA does not directly interact with the cyclodextrin. This process is fast and comparison of the molar ratio required for a complete transfer of the fatty acid to the protein indicates that the total quantity of the iodinated fatty acid is extracted from the complex when ca. 1/7 molar equivalent of the protein is added. This implies that the HSA molecule has at least 7 sites for the fatty acid with affinities far above the value found for the CD-fatty acid interaction. This value is in excellent agreement with data found in the literature concerning the association of serum albumin with linear fatty acid where at least 7 strong sites are evidenced by Scatchard plots [6–8].

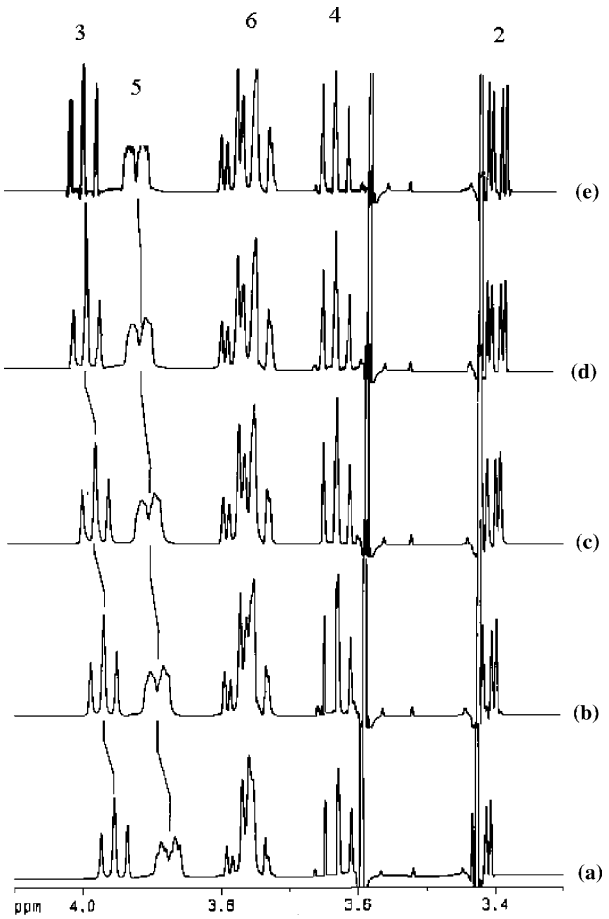

Figure 3. Partial 500 MHz ^{1}H NMR spectra of 3 mM DIMEB and 1.25 mM (16-iodo-3-methylhexadecanoic acid) in the absence (a) and in the presence of 37 μM (b), 92 μM (c) and 184 μM (d) human serum albumin (HSA) The top spectrum (e) is obtained with a sample containing 3 mM DIMEB and 184 μM HSA. Experimental conditions are in Figure 1.

Conclusion

This study shows that modified cyclodextrins are excellent candidates to make radiolabeled fatty acid soluble used for diagnostic purposes. NMR has allowed a better understanding of the structure and affinity (strength) of the formed inclusion complex. Moreover, a detailed investigation of the competition between this carrier molecule and the putative transporter (HSA) has shown that upon injection, the included molecule is taken out

from the cavity and loaded into the neutral transporter. An increase in the quality of image might be related to the protection effect of the solubilization process using modified cyclodextrins. The partial degradation of the carrier protein resulting in the exposure of unexpected sites leads to an irreversible binding (after heating the radiolabeled compound in the case of the solubilization with the HSA as adjuvant).

This new formulation allows for a sterilization cycle according to the European Pharmacopoeia.

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